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Page 100, line 9, "less virulence" should read "loss of virulence."

Page 137, Table X, Plot L, "Nitrate of soda" should read "Nitrate of soda, dried blood, and sulphate of potash."

Page 164, Pl 3, A, "250" should read "25°."

Page 198, lines 3 and 5, "B" should read "C" and "C" should read "B"

Page 198, line 9, "0.1 c. c." should read "0.01 c c."

Pages 206, 208, Tables V and VI, "test" should read "soil."

Page 237, line 3 from bottom, "microscopic" should read "macroscopic"

Page 265, line 5 from bottom, "Pearl (22) says" should read "Pearl (21a) says".

Page 275, insert "(21a) Pearl, Raymond. Modes of Research in Genetics. 182 p., diagr. New York, 1915."

Page 275, (22), "Bul 100" should read "Bul 110"

Page 329, Table I, under "Host," "*Peltandra virginicia*" should read "*Peltandra virginica.*"

Page 364, line 23, "(21)" should read "(5)."

Page 374, Table VIII, footnote "a" should refer to "Experiment 3" only

Page 393, line 16, "Petite" should read "Petit"

Page 409, line 3 from bottom, "as finely granular structure as in *B. mesentericus*" should read "as finely granular in structure as *B. mesentericus.*"

Page 413, line 7 from bottom, "To this, bee-larvæ bouillon," omit comma after "this."

Page 422, line 25, "(13, 14), and Manns" should read "(14), and Manns (13)."

Page 422, line 30 "(fig. 1, A–G)" should read "(fig. 1, H),"

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DIAGNOSIS OF TUBERCULOSIS BY COMPLEMENT FIXATION, WITH SPECIAL REFERENCE TO BOVINE TUBERCULOSIS

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INTRODUCTION

Ever since the nature of tuberculosis has been established, the diagnosis of the disease in living animals as well as in man has caused considerable difficulty. Therefore it is not at all surprising that the efforts of the investigators were directed to devise means by which this most destructive disease could be readily and with certainty diagnosed. With the discovery of tuberculin a great advancement was made in this direction, especially with regard to its diagnostic value in animals. Nevertheless its shortcomings have been recognized, not because of its being unreliable but on account of the laboriousness of its application and the possibility of the so-called "doping" of the animals for the purpose of preventing a reaction. This practice is known to have been quite extensively employed in this country and in Germany, where the authorities were forced to abandon the recognition of this wonderful test on border importation as a result of the frequent frauds. In the search for other reliable means for diagnosis other allergic tests have been employed, such as the ophthalmic, cutaneous, intradermal, and intrapalpebral tests; but while favorable reports have been made on these various tests by individual investigators, as a whole they do not come up to the reliability of the subcutaneous test when it is scrupulously carried out.

The success obtained with the various biological tests for the diagnosis of infectious diseases directed attention toward their application in tuberculosis. Of the different biological determinations the agglutination and precipitation tests held out no encouragement for success. On the other hand the good results obtained with the complement fixation method for

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the diagnosis of diseases of animals, such as glanders, dourine, contagious abortion, Malta fever, etc., seemed to warrant further investigations for establishing the value of the test in tuberculosis, especially since the published results on similar investigations are very contradictory.

HISTORICAL RÉSUMÉ

It is well known that the first practical application of the Bordet-Gengou (6)[1] reaction was undertaken by Widal and Lesourd (40), who showed that antibodies of typhoid infections may be established much earlier in the serum of the patient by means of the complement fixation than with the agglutination method. Notwithstanding these clinical observations and the great possibilities for the Bordet-Gengou reaction, it was neglected for years until it was again taken up by Moreschi (29). This theoretical conception as to the reaction was not acceptable, especially since the investigations of Wassermann (37), Bruck (9), Liefmann (25), and Citron (12) showed, and later Moreschi himself recognized, that his former views could not be supported by the results of the subsequent investigations. According to Moreschi's theory the Bordet-Gengou reaction represented a precipitation reaction, and the formed precipitin was mechanically carried down by the complement. When he later observed that at times even strongly precipitating serums failed to give the reaction, he recognized the unsoundness of his theory.

Neisser and Sachs (31) also undertook investigations with the Bordet-Gengou reaction, endeavoring to employ it for the differentiation of albumens. Since their findings were irregular and contradictory, Wassermann carried on investigations to establish whether it would be possible to differentiate dissolved bacterial albumens with the aid of the test. Wassermann further aimed to prove that before the state of antibody production there are probably other intermediate products in the circulation which might be detected by means of complement fixation as applied by Neisser and Sachs for the differentiation of albumens. Wassermann failed to demonstrate the presence of such intermediate bodies; and in collaboration with Bruck (37) he directed his attention toward establishing the presence of an antigen in diseased organs, using lungs, glands, and other diseased tissues affected with tuberculosis. Extracts of these organs were prepared and used against serums of tuberculous individuals, with the result that such serums did not fix the complement unless the patient had been treated with tuberculin for a certain period. The reaction obtained was due to the formation of antibodies as a result of the injection of tuberculin. Wassermann called these antibodies "antituberculin." Citron (12), on the other hand, named them "antituberculin amboceptors."

The findings of Wassermann and Bruck were questioned by various investigators. Weil and Nakayama (38) and Morgenroth and Rabino-

[1] Reference is made by number to "Literature cited," p. 18–20.

witz (30) were the first who disagreed with their conclusions. Citron, while agreeing with the principles of the Wassermann-Bruck conception, continued the investigations for the purpose of determining whether it would be possible to establish antibodies in the blood of tuberculous individuals, though they were not treated with tuberculin. Employing Wassermann's method, he obtained in 9 untreated cases of tuberculosis 2 positive and 7 negative results; whereas in 14 treated cases, 8 were positive and 6 negative. Ludke (27) also confirmed the essentials of the Wassermann-Bruck work, as he found antituberculin present in tuberculous tissue in man in 3 out of 5 cases. He also found it in the serum in 17 out of 31 treated cases and in 2 advanced cases out of 10 untreated cases. In 18 cases, none of which were tuberculous, negative results were obtained in all instances. Ludke also observed that antituberculin was present, especially in cases where the administration of tuberculin was not followed by a systemic reaction. In the cases in which this injection was followed by a reaction he failed to obtain any fixation. In 8 cases tested both before and after the administration of tuberculin antibodies were found in 7 after injection. In 3 cases affected with diseases other than tuberculosis the injection of tuberculin was not followed by the appearance of antituberculin.

Wolff-Eisner (42) denied the specificity of the reaction because they found that the serums of patients suffering from pneumonia, typhoid fever, or lues gave a similar reaction to those affected with tuberculosis. Laub and Novotny (23) also questioned the usefulness of the reaction, as they found that the results were very irregular, tuberculous patients giving the same proportion of reactions as healthy individuals. Cohn (13), on the other hand, established the usefulness of the reaction, provided the test is carried out with the utmost care, including the use of all controls, in which case the presence of antibodies may be demonstrated not only by the tuberculin-treated individuals but also in progressive cases. A number of investigators, as M. Wolff and Mühsam (41), Czastka (15), Bernbach (2), Weil and Strauss (39), claimed to have been able to obtain favorable reactions in 20 to 50 per cent of their tests. On the other hand, the followers of Escherich (20) declared that at no time was it possible to determine antibodies in tuberculous children. Schlossmann (34) and Engel and Bauer (19) confirmed these views. Engel and Bauer, investigating a series of tuberculous affections in children, failed to find specific antibodies either in the serum of healthy or in infected children. In all cases of tuberculosis which were specifically treated, antibodies developed. They further found that the antibodies increased in proportion to the injections of tuberculin and diminished after discontinuing the treatment. Frugoni (21) and Szabóky (36) also concluded that the complement fixation in tuberculosis is of no value for diagnostic purposes, which view was also held by Bach (1) with regard to animal tuberculosis.

These contradictory results stimulated the investigators to further work. Dieterlen (17), continuing the experiments of Christian and Rosenblat (11), found that normal animal serums do not contain antibodies but that spontaneously or artifically infected animal serums possess them. Szabóky stated that rabbits always possess antibodies against tuberculin, but that they disappear whenever the animal becomes tuberculous. In guinea pigs such antibodies are invariably absent. Löwenstein (26), employing old and new tuberculin as antigen, found antibodies present in a high percentage of tuberculous individuals in advanced stages of the disease. In cases of recent infections they were almost invariably absent. In cases treated with tuberculin they usually disappear for a short time and reappear about the eighth day after the injection and subsequently increase in amount. Ruppel (33) noted a marked rise in the quantity of antibodies in the serums of bovines inoculated with human tubercle bacilli and treated with tuberculin.

Emery (18) employed sterile bacillary emulsion as antigen, using the complement present in the serum to be tested, and human red blood corpuscles, also a special technique by varying the time during which the ingredients remained together at 38° C. He obtained 82 per cent of positive reactions in 56 cases of tuberculosis and 17.6 per cent positive reactions in 34 nontuberculous cases.

Porter (32) investigated the sera of cattle and obtained complement fixation in normal as well as in tuberculous cases, the reaction being more characteristic in the advanced cases than in the early stage of the disease.

Wyschelewsky (43), also working with cattle, confirmed Porter's results. Both observers concluded, however, that this method is not suitable for the differentiation of latent cases from the more progressive ones.

Deilmann (16) compared the various extracts from the tubercle bacilli such as proteins, fatty acid, and tuberculo-nastin individually and also each in turn with tuberculin for their antigenic properties, and concluded that all the extracts act as antigens, but none are as suitable in this respect as an emulsion of the entire bacillus or as tuberculin.

Calmette and Massol (10) and also Letulle (24) used as antigens watery extracts of bovine tubercle bacilli and bacilli macerated in peptone. They followed a more minute technique by adding varying doses of complement and succeeded in demonstrating antibodies in a large percentage of tuberculous cases. They consider that antibodies are not invariably present, because in many cases a substance is present in the serum which inhibits the reaction. They further obtained the most marked reactions in cases of a slow progressive character, while in acute cases the results were more often negative.

Hammer (22), in using a mixture of tuberculous synovial membrane and tuberculin as antigen, obtained out of 96 cases, 50 positive and 46

negative reactions, of which 48 were found positive and 48 negative on post-mortem examination. Exception was taken to his findings by Bierbaum and Berdel (5), who concluded that alcoholic or aceton extracts of tuberculous tissues as antigens do not confirm the results of Hammer. Of all antigens the greatest support was given to the one prepared by Besredka (3, 4). It is essentially a tuberculin prepared in glycerin and peptone-free egg medium. Many investigators reported favorably on the action of this antigen, and the only objection against it was its nonspecific reaction obtained in cases of nontuberculous syphilitic individuals. Bronfenbrenner and Rockman (8) concluded with regard to Besredka's antigen that different samples of his tuberculin, though prepared apparently in an identical manner, may differ in their specific values. Thus, different samples were found to vary in the amount of lipins they contained. It is necessary to free the samples of tuberculin entirely from the lipins present when used for the complement deviation test. Besredka's antigen seems to give the best results in the diagnosis of tuberculosis by means of complement fixation, and though the test is positive in a certain number of nontuberculous cases the reaction seems to be specific. However, some investigators experienced difficulties in preparing the antigen according to the directions given by Prof. Besredka.

More recently Stimson (35) carried on extensive investigations on this subject and employed an alcoholic precipitate of the tubercle bacillus emulsion filtrate as antigen, obtaining promising results. Favorable results were further reported by Craig (14) with an antigen prepared from a liquid egg-medium growth material, and by Miller and Zinsser (28) with a bacterial emulsion in hypotonic salt solution. These authors claim good results, especially for differentiation between active and nonactive infections.

The foregoing historical outline furnishes ample evidence that strong efforts were made toward devising a method by which the complement fixation might be utilized for the diagnosis of tuberculosis

ANTIGENS

In the foregoing literature mention was made of the various antigens which were used for the diagnosis of tuberculosis by means of complement fixation. These antigens may be classified into three groups, according to their contents:

1. Tuberculin.
2. Emulsion of tubercle bacilli.
3. Extracts of tuberculous tissues.

In consideration of the failures of former investigators with various antigens containing the specific products of the germ and of the disease, it suggested itself that an antigen containing the toxic products together with bacteria would probably combine the active elements for the phenomena of the reaction. Therefore experiments were undertaken with

an antigen consisting of tuberculin and tubercle bacilli in varying proportions.

In the preparation of tuberculin the liquid medium contains glycerin in an amount varying from 5 to 7 per cent. Besredka, in preparing his egg medium, eliminated entirely the glycerin. In the following experiments the ordinary old tuberculin freed from its glycerin contents has been utilized. The bacillary emulsion used by different authors was prepared in various ways. The emulsions consisted of finely triturated bacteria suspended in normal or hypotonic salt solution. Alcoholic precipitates of bacterial extract were also utilized (14, 35). For the purposes of the experiments only defatted organisms were employed, because it was assumed that the waxy capsule of the bacilli might prevent the extraction of the endotoxins; and furthermore, it was desired to avoid nonspecific reactions due to the presence of lipins. Therefore the antigen was composed essentially of concentrated tuberculin and the defatted organisms. At the same time comparative tests were carried out with other antigens. The following are especially mentioned:

1. Emulsions of tubercle bacilli in normal or hypotonic salt solutions.
2. Emulsions of defatted tubercle bacilli in normal or hypotonic solutions.
3. Unheated nonconcentrated tuberculin freed from bacilli by passage through Berkefeld filters.
4. Unheated nonconcentrated tuberculin freed from bacilli by passage through Berkefeld filters fortified with aceton insoluble lipoids, as prepared by Noguchi. The aceton insoluble lipoids were previously standardized against known positive syphilitic sera to establish its titer. One unit of the antigen was added to 10 c. c. of the tuberculin mentioned and this mixture was then used as a menstruum in the emulsification of the bacilli.
5. Besredka's antigen—(a) as received through the courtesy of Prof. Besredka, (b) a modification of the same, (c) a modification of the same unheated and freed from bacilli by passage through Berkefeld filters.

The modified Besredka's medium was prepared as follows: Twenty c. c. each of the white and the yolk of an egg were thoroughly beaten in an automatic egg beater, and to the whipped material a solution of Liebig's meat extract (3:1,000) in distilled water was gradually added while the mixture was continuously beaten. A sufficient meat-extract infusion was used to make up the whole to 1,000 c. c. The emulsion was strained through cotton and heated to the boiling point, then strained again, and after the addition of 0.5 per cent of sodium chlorid was carefully neutralized, heated again and strained, and neutralized if necessary. To the neutral medium sufficient normal sodium-hydroxid solution was added to make the medium of 0.2 alkalinity. This medium, to which neither peptone nor glycerin was added, was then autoclaved at 115° C. for 20 minutes, and after cooling was kept at 37° for 48 hours for observation as to sterility. Human and bovine cultures containing a 30-days' growth of the tubercle bacilli, heated for 20 minutes at 115° and filtered, were used as antigen. This antigen was standardized in the usual manner.

EXPERIMENTAL WORK

PREPARATION OF THE ANTIGENS EMPLOYED

Material used: Old tuberculin; saturated solution of ammonium chlorid; Schulke and Schleich parchment paper.

To an amount of old tuberculin in an Erlenmeyer flask of convenient size sufficient saturated solution of ammonium chlorid is added to supersaturate the tuberculin. The mixture is stirred well with a glass rod and set aside for 48 hours. The fluid is carefully poured off from the precipitate which has settled at the bottom of the flask. The liquid is centrifuged at a high rate of speed and the sediment collected, which consists of a resinous sticky mass, dark brown in color, is readily dissolved in a small amount of salt solution, and is then transferred to the dialyzing (parchment paper) bag. The sediment contained in the Erlenmeyer flask is added to it and the material is then dialyzed for 8 consecutive days. The content of the bag is then tested for the presence of ammonium chlorid, and if no reaction for ammonia is obtained the substance is removed, filtered, and the filtrate evaporated to dryness over a water bath at 60° C.

The residue was scraped from the evaporating dish, and the chocolate-brown colored powder collected, placed in a bottle which was provided with a well-fitting stopper, and the precipitate kept in a cool place, protected from light. The amount of precipitate obtained corresponds to about 0.8 to 1 per cent.

It should be emphasized that the preparation and handling of the precipitate must be carried out under precautions to prevent the inhalation of the pulverized precipitate. Even slight amounts may cause disagreeable symptoms. These are manifested in severe headaches and chills, accompanied by high fever, the temperature ranging between 102° and 104° F. for two or three consecutive days; pulmonary symptoms resembling those of a lobular pneumonia and profuse night sweats, respiration sometimes difficult and painful; pains in the back are always present; the urine is dark-colored; micturition frequent and scanty. For protection it is advisable to cover the evaporating dish during the pulverization with several layers of cheesecloth moistened with a 5 per cent phenol solution.

The material used for the preparation of defatted organisms was as follows: Surface growth of a bouillon culture of tubercle bacilli of both human and bovine strains; alcohol, 95 per cent; ether.

The tubercle bacilli are macerated for seven days in 95 per cent ethyl alcohol in a closed vessel. The alcohol is then removed and the bacteria are macerated with 50 c. c. ethyl ether for six hours, shaking the mixture frequently. The ether is poured off and the bacilli are shaken two more times for one-half hour each time with 25 c. c. of ether. The bacilli are then collected, dried in vacuum over sulphuric acid, and

finally kept in a well-stoppered vial in a cool place protected from the light.

Standardization of Antigen for Final Test

Complement.—In the early work of the writers on the complement fixation in tuberculosis the intention was to use one unit of the accurately standardized complement. The titer was determined by establishing the smallest amount of complement which caused complete hemolysis within one-half hour of incubation. It was soon observed, however, that this amount was insufficient to establish the proper amount of complement necessary to obtain the best results. It was found that a 5 per cent dilution proved most suitable, and accordingly that proportion was employed throughout this work.

Two units of anti-sheep-blood hemolysin were used throughout.

Red sheep-blood corpuscles.—Various dilutions of the suspension were tested, and the most satisfactory results were obtained with a 3.5 per cent sheep-blood corpuscle.

Salt solution.—While the normal physiologic salt solution was used in most cases, tests were also made with 0.75 per cent. It was noted that by employing the hypotonic solution the reactions developed more rapidly.

Serums used for standardization of antigens.—The serums were obtained from tuberculin-tested animals. At least 12 months elapsed from the time of the last negative test. The inactivation was carried on at 58° C. for one-half hour and 0.2 c. c. was the amount used in the test in every tube.

Two methods were used in the standardization of the antigen.

Method 1: Standardization of Antigen

Thirty mgm. of defatted and dried tubercle bacilli were pulverized in a 12-cm. mortar. The powder was then emulsified in a solution containing 50 mgm. of dried tuberculin in 10 c. c. of 0.6 per cent salt solution. This emulsion served as the antigen and was titered for its antigenic, anticomplementary, and hemolytic properties. The titration was conducted according to Tables I to III.

Table I.—*Titration for antigenic dose*

Tube No	Antigen	Inactivated positive serum	Complement, 5 per cent	Physiological salt solution	Incubation period	Hemolysin	Erythrocytes, 3.5 per cent	Incubation period	Degree of hemolysis
	C c	C c	C c				C c		
1	0.5	0.2	1	Sufficient quantity to make 2.0 c. c.	1 hour.	2 units contained.	1	1 hour	None
2	.4	.2	1			do.	1		Do.
3	.3	.2	1			do.	1		Do.
4	.2	.2	1			do.	1		Do.
5	.1	.2	1			do.	1		Incomplete.
6	.05	.2	1			do.	1		Complete.
7	None.	.2	1			do.	1		Do.
8	.1	None.	1			do.	1		Do.

TABLE II.—*Titration for anticomplementary dose*

Tube No.	Antigen	Inactivated normal serum.	Complement, 5 per cent.	Physiological salt solution.	Incubation period	Hemolysin	Erythrocytes, 3 5 per cent	Incubation period.	Degree of hemolysis
	C c	C c	C c				C c		
1	0. 5	0 2	1	Sufficient quantity to make 2 c. c	1 hour	1 c. c. containing 2 units.	1	1 hour	Incomplete.
2	.4	.2	1			.do.....	1		Complete.
3	.3	.2	1			.do... ..	1		Do.
4	.2	.2	1			do...	1		Do.
5	.1	.2	1			do...	1		Do
6	.005	.2	1			do	1		Do
7	None.	.2	1			do	1		Do
8	.1	None				do	1		Do

TABLE III.—*Titration for hemolytic properties*

Tube No	Antigen	Inactivated normal serum	Erythrocytes	Physiological salt solution	Incubation.	Degree of hemolysis.
	C c	C c	C c			
1	0 6	0 2	1	Sufficient quantity to make 3 c c	1 hour	Very faint
2	5	.2	1			None
3	4	.2	1			Do
4	3	.2	1			Do
5	.2	.2	1			Do
6	1	.2	1			Do
7	05	.2	1			Do
8	None					Do
9	3	None				

METHOD 2: STANDARDIZATION OF BACILLARY EMULSION WITH A FIXED AMOUNT OF TUBERCULIN PRECIPITATE

Solutions containing 10, 20, 30, 40, etc., mgm., in 10 c. c. of a 0.6 per cent solution of sodium chlorid were prepared from the tuberculin precipitate described under the preparation of antigen. Tests were undertaken with the various dilutions, 0.1 c. c. of each serving as a basic amount of the antigen to which increasing amounts of the bacillary emulsion had been added in the different tubes. Tables IV to VI demonstrate the procedure of the tests.

TABLE IV.—*Titration for antigenic dose*

Dose No.	10 mgm tuberculin precipitate in 10 c. c sodium-chlorid solution.[a]	Bacillary emulsion	Inactivated positive serum	Complement, 5 per cent.	Physiological salt solution	Incubation	Hemolysin (1 unit = 0 5 c c)	Erythrocytes, 3 5 per cent	Incubation	Degree of hemolysis
	C c	C c	C c	C c				C c		
1.	[b] 0. 1	0. 40	0 20	1	Sufficient quantity to make 2 c c	1 hour	2 units	1	1 hour	None
2	[b] 1	.30	.20	1			do	1		Do
3.	[b] .1	.20	20	1			do. .	1		Do
4	.1	.15	20	1			do	1		Do
5	1	10	.20	1			do .	1		Incomplete
6	.1	.05	20	1			do	1		Complete
7	.1	None	20	1			do	1		Do
8	.1	.10	None.				do .	1		Do

[a] Similar tests were made with solutions containing 20, 30, 40, and 50 mgm. of tuberculin precipitate in 10 c. c. of a solution of sodium chlorid

[b] In solutions 1, 2, and 3, four mgm. per cubic centimeter were used.

TABLE V.—*Titration for anticomplementary dose*

Dose No.	Tuberculin precipitate solution.	Bacillary emulsion.	Inactivated normal serum	Complement, 5 per cent.	Physiological salt solution.	Incubation period.	Hemolysin (1 unit = 0.5 c. c.)	Erythrocytes, 3.5 per cent	Incubation period.	Degree of hemolysis.
	C. c.	*C. c.*	*C. c.*	*C. c.*				*C. c.*		
1.....	0.1	0.40	0.2	1			2 units	1		Incomplete
2.....	.1	.30	.2	1			do.. .	1		Complete
3.....	.1	.20	.2	1			do..	1		Do.
4.....	.1	.15	.2	1			do....	1		Do.
5.....	.1	.10	.2	1			do .	1		Do.
6.....	.1	.05	.2	1			do .	1		Do
7.....	.1	None	.2	1			do	1		Do
8.....	.1	.1	None	1	Sufficient quantity to make 2 c. c.	1 hour.	do	1	1 hour.	Do.

TABLE VI.—*Titration for hemolytic properties*

Tube No.	Solution of tuberculin precipitate	Bacillary emulsion.	Inactivated serum	Erythrocytes, 3.5 per cent	Physiological salt solution	Incubation period.	Degree of hemolysis
	C. c.	*C. c.*	*C. c*	*C. c*			
1.. . .	0.1	0.40	0.2	1			None
2.. .	.1	30	.2	1			Do
3 . .	.1	.20	.2	1			Do.
4 . .	.1	.15	.2	1			Do.
5	.1	.10	.2	1			Do
6 ...	.1	.05	.2	1			Do
7	.1	None.	2	1			Do
8	1	15	None	1	Sufficient quantity to make 3 c. c.	1 hour.	Do
9	1	None	.2				Do

Table VII demonstrates the procedure employed in the final test and also the controls used.

TABLE VII.—*The final test*

Tube No.	Antigen	Inactivated serum 0.2 c. c.	Complement, 5 per cent.	Physiological salt solution.	Incubation period at 37° C.	Hemolysin (1 unit = 0.5 c. c.)	Erythrocytes, 3.5 per cent	Incubation period at 37° C.	Degree of hemolysis
Positive controls.	*Unit.*		*C. c.*	Sufficient quantity to make 2 c. c.	1 hour	*Units.*	*C. c.*	1 hour.	
1 .	1	+ Serum	1			2	1		None
2	1½	do	1			2			Do
3 .		do	1			2	1		Perfect.
Negative controls									
4	1	− serum	1			2	1		Do
5	1½	do	1			2	1		Do.
6		do	1			2	1		Do.
Suspected serum:									
7	1	Suspected serum	1			2	1		?
8	1½	do	1			2	1		?
9	.	do	1			2	1		?
Controls									
10	1½	do	1			2	1		Perfect
11			1			2	1		Do

SOURCE AND TESTING OF SAMPLES

The material for testing was obtained from animals slaughtered at the Baltimore and the Washington slaughterhouses and from animals which were subjected to periodical tuberculin tests. The samples obtained from the abattoirs were collected in sterile glass jars or test tubes. Every sample of blood was accompanied by a post-mortem report from the veterinary inspector, describing in detail the condition of the animals and also the character and extent of the lesions. Figure 1 is a repro-

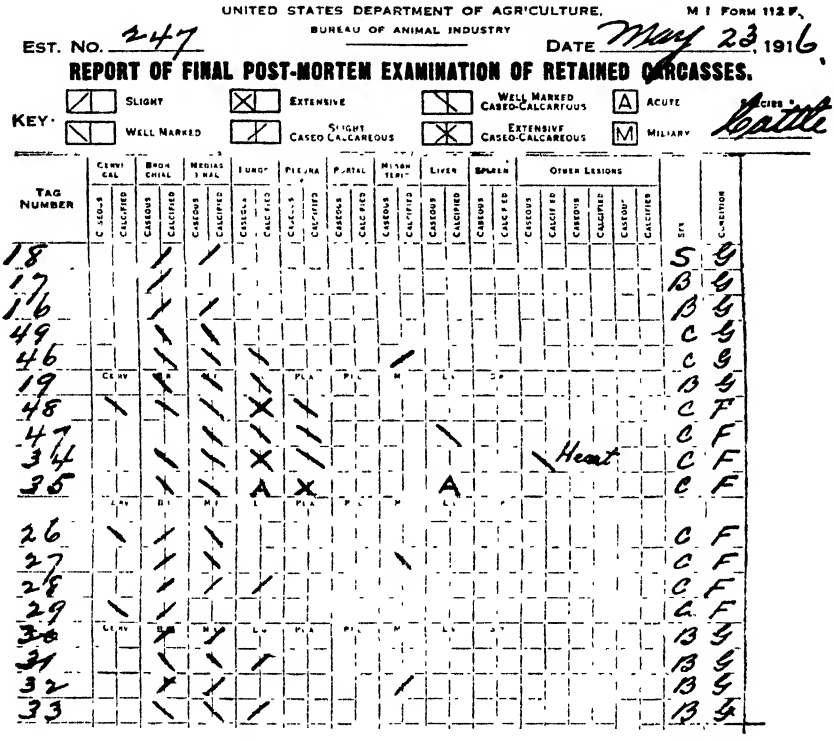
UNITED STATES DEPARTMENT OF AGRICULTURE. M I Form 112 F.
BUREAU OF ANIMAL INDUSTRY
EST. NO. 247 DATE May 23, 1916.
REPORT OF FINAL POST-MORTEM EXAMINATION OF RETAINED CARCASSES.
KEY: Slight; Well Marked; Extensive; Slight Caseo-Calcareous; Well Marked Caseo-Calcareous; Extensive Caseo-Calcareous; Acute; Miliary
Species: Cattle
Tag Number | Cervical | Bronchial | Mediastinal | Lungs | Pleura | Parietal | Miliary Tbc. | Liver | Spleen | Other Lesions | Sex | Condition
18 S G
17 B G
16 B G
49 C G
46 C G
19 B G
48 C F
47 C F
34 C F
35 C F
26 C F
27 C F
28 C F
29 C F
30 B G
31 B G
32 B G
33 B G

FIG. 1.—Specimen of post-mortem report on retained carcasses in Federal meat inspection

duction of a post-mortem report showing the character of the lesions of animals the serums of which were subjected to the complement-fixation test.

The samples of blood obtained from animals prior to the injection of tuberculin were conveyed from the jugular direct into sterile bottles. All samples of blood were delivered at the laboratory without any delay. After the breaking up of the clots, they were centrifuged; and from the supernatant clear serum a sufficient quantity was taken for the test.

Some tests were performed with active serums, but without success, as the results in most instances showed an anticomplementary action.

Inactivation at 55° for one-half hour was not uniformly satisfactory; it was found that an inactivation of 58° gave the best results. Buck's method of inactivation (unpublished) was also employed, in which equal parts of salt solution are added to the serums and the inactivation is carried at 62°. Both methods were uniformly satisfactory, though in a few instances Buck's method appeared to have an advantage over the former. The quantity of the inactivated serum used for the test was 0.2 c. c.

Table VII describes in detail the procedure of the test, in which the incubation time is designated as one hour. In order to obtain the best results in the preliminary experiments, care was taken to establish the length of time the racks should be kept at incubation before adding the hemolysin and the suspension of blood corpuscles. By subjecting various racks to incubations from one to four hours it was found that one-hour periods served to the best advantage.

RESULTS OF TESTS

In all 958 samples were tested, of which 816 samples were of bovine, 120 of porcine, and 22 of human origin. A summary of the tests is contained in Table VIII, which also describes the lesions found on post-mortem examination of the cattle in the abattoirs.

In order that a clearer conception might be obtained as to whether there exists any relation between the degree of reaction and the character of the disease it was deemed advisable to separate the cases into five groups according to the lesions found on post-mortem examination, as follows:

1. Animals which showed no lesions of tuberculosis and which failed to react to the tuberculin test.
2. Animals which showed arrested lesions with a limited number of small slight caseo-calcareous foci confined to the lymph glands.
3. Animals with progressive lesions of a glandular type involving also some of the organs.
4. Animals with well-marked or generalized lesions.
5. Acute and miliary tuberculosis.

In considering the results of these tests there appears to be no constancy in the reaction; and furthermore, the degree of the reaction appears to have no relation to the extent of the disease. It was also noted that the condition of the animal has apparently no effect on the reaction, which as a matter of fact is also the case in the subcutaneous tuberculin test, although in the latter advanced generalized cases may fail to respond.

The degree of the fixation is also irregular, since the slight positive reactions (+) do not occur proportionally with a greater frequency in mild cases than the complete fixation (+ + + +). This must be considered a great disadvantage, as a slight reaction might be overlooked or might be obtained as a result of the slightest neglect in the standardization of the

antigen. It may be possible that future investigation will establish a greater accuracy of this test, but the results obtained warrant the statement that the comparative tests with various antigens have proved that an antigen prepared from a bacillary emulsion and tuberculin precipitate is most suitable for the completement-fixation test.

TABLE VIII.—*Summary of the tests conducted with an antigen prepared from a bacillary emulsion and tuberculin precipitate*

Bovine serum.	Total number.	Degree of fixation						Percentage
		+ + + +	+ + +	+ +	+	±	−	
Group 1 Serums from animals without lesions of tuberculosis and negative to the tuberculin test	320	3	2	10	13	17	275	85.94
Group 2 Serums from animals showing arrested lesions of tuberculosis	207	21	28	39	48	19	52	77.15
Group 3 Serums from animals with progressive lesions	79	11	16	23	16	5	8	83.56
Group 4 Serums from animals showing generalized, well marked, or extensive lesions	43	12	9	4	7	4	7	74.42
Group 5 Serums from animals showing acute or miliary lesions	31	9	4	4	10	1	3	87.1
Total	680	56	59	80	94	46	345	81.63

As indicated in Table VIII, out of 320 negative serums a total of only 275, or 85.9 per cent, proved distinctly negative on the test; whereas of 360 positive serums 290, or 80.5 per cent, were positive. Furthermore, of the affected animals 81, or 25.8 per cent, gave only slight positive reactions, and 80, or 7.6 per cent, were atypical. The grand total of 81.6 per cent of accurate reactions obtained is by no means sufficient to make the test practical for the control of the disease, especially since the allergic tests which we have at our command are more reliable.

No satisfactory explanation can be given for the failure of reactions in known tuberculous infections or for the positive reactions in apparently healthy individuals. In regard to this various theories have been advanced; but since there is no confirmatory data available for any of these, it appears superfluous to discuss them here. The fact, however, that there existed a possibility that some of the positive results in the negative cases might have been due to a previous tuberculinization of the animals can not be overlooked, since no history was available regarding the animals which furnished the serums. It is therefore probable that a number of the atypical and possibly also some of the positive reactions in the apparently healthy animals might have resulted from an injection of tuberculin, which to-day is quite extensively practiced for diagnostic purposes.

In order to establish whether a tuberculin injection would interfere with the results of the tests, two vigorous young animals were subcutaneously injected with 2 c. c. of Bureau of Animal Industry tuberculin. Blood samples were obtained from these animals prior to the injection and subjected to the complement-fixation test with negative results. Subsequent to the tuberculinization, blood was drawn twice weekly from them. On the fourth day following the injection a positive reaction was obtained; after 10 days the fixation was complete, persisting for four weeks; it was then followed by partial fixation, and completely subsided after six weeks. It is possible that in some animals the fixation might persist for a still longer period; therefore it is essential to determine whether an animal has been injected with tuberculin, and if so, how long ago, before judgment can be passed on the results of the complement-fixation test.

In connection with these experiments a peculiar phenomenon was observed in several instances. In using an antigen which contained a higher amount of precipitate than the standard established by titration, a reaction which might be designated as a reverse reaction followed—that is, instead of the expected positive reaction it proved negative, and vice versa.

While it is not intended to offer a theoretical explanation for this peculiarity, it serves to emphasize the necessity for a careful standardization of the antigen each time the test is undertaken. The apparent delicacy of the test in the diagnosis makes it essential to employ more than one known positive and known negative serum in standardizing the antigen. In the experiments cited the antigens were always standardized to a series of known positive and negative serums.

COMPARATIVE TESTS WITH VARIOUS ANTIGENS

The advantages of an antigen prepared by our method have already been described. In order, however, to establish its comparative value various other antigens were used and the following brief mention is made of the methods of their preparation and the results obtained.

For the antigen prepared from tubercle bacillary emulsion, dilutions were made from a stock emulsion in which 30 mgm. were suspended in each cubic centimeter of the emulsion. The stock suspension was never heated and was prepared by milling the bacilli in a porcelain mill for about 40 days. The dilution used as antigen consisted of 3 mgm. per cubic centimeter in either physiological salt solution or in hypotonic solution. It was observed that the emulsion made up with the normal physiological salt solution gave an excessive number of positive reactions, whereas the one prepared with the hypotonic solution gave much closer results. Nevertheless the comparative results proved this antigen to be unsatisfactory. Its greatest disadvantage proved to be that the difference between the

antigenic and the anticomplementary properties was of such short range that the reliability of the tests with this antigen had to be questioned.

The same experience was obtained with an antigen prepared from defatted organisms. The only advantage of this antigen as compared with the preceding one was noted to be in the shorter time in which the reaction took place, and in some instances it appeared more reliable. The precipitation method of Stimson (35) was also tried, but without much success. It was the intention also to employ the combined Besredka-Stimson method, as advocated by Craig (14), but this was discarded when a communication was received from Dr. Craig in which he stated that the antigen devised by him might be improved.

An antigen prepared from an unheated liquid medium and Berkefeld-filtered proved to be of no value. The same in combination with the bacteria gave results similar to the antigen prepared from tubercle bacillary emulsion and from defatted organisms; and when reinforced with lipoids, the reactions obtained were much more distinct. At the same time, however, many nonspecific reactions were obtained. According to Calmette (10), the serum of a tuberculous individual contains more lecithin than that of a healthy person. Whether this observation has any bearing on the more definite reaction obtained with antigens containing a lipoid substance or whether the more distinct reactions are due to changes of lipoidic bodies present in the serum can not be stated.

In using Besredka's antigen for comparative tests it was desirable to establish whether the nonspecific reaction reported by Bronfenbrenner (7) and Stimson (35) could be observed in tests conducted with serums of cattle. In these instances the presence of syphilis, which was assumed to be the cause of some atypical reactions, was excluded. Nevertheless similar disturbing factors were observed in the tests with bovine serums. It has been stated that some of the nonspecific reactions might have been due to the possibility that the serums originated from animals which had been previously tested with tuberculin. There were, however, nonspecific reactions obtained in animals belonging to a herd in which the tuberculin test had not been applied for at least one year, the animals of this herd having been proved free from tuberculosis for years by periodical testing. Table IX serves as a comparison as to the results obtained with Besredka's antigen and the one prepared by the writers from tuberculin precipitate and defatted organisms.

The number of cases tested and the results recorded in Table IX would not appear to justify any definite claims as to the comparative value of Besredka's antigen and the one prepared by the writers. However, in the course of these experiments numerous samples of serums were repeatedly tested with various antigens in order to establish their relative accuracy, and, based especially on these results, a considerable advantage appeared to be in favor of the antigen prepared from a bacillary emulsion and tuberculin precipitate.

TABLE IX.—*Summary of the tests conducted for comparative purposes with (I) the antigen of Besredka and (II) the antigen prepared from a bacillary emulsion and tuberculin precipitate*

Bovine serum.	Number of cases.	Degree of fixation.												Percentage.	
		++++		+++		++		+		±		−			
		I	II	I	II	I	II	I	II	I	II	I	II	I	II
Group 1 Serums from animals without lesions of tuberculosis and negative to the tuberculin test	91	2	.....	1	2	1	1	4	3	....	..	81	83	91.1	93.4
Group 2 Serums from animals showing arrested lesions of tuberculosis..	35	2	.....	...	....	1	.	12	19	4	7	16	9	42.80	54.29
Group 3 Serums from animals showing progressive lesions of tuberculosis..	3	...	...	....	..	...	....	2	3	1	...	.....	.....	66.67	100
Group 4 Serums from animals showing generalized well marked or extensive lesions ..	1											1	1		.
Group 5 Serums from animals showing acute and miliary lesions of tuberculosis	6					1	1			4	4	1	1	16.35	16.35
Total	136	4		1	2	3	2	18	25	10	11	99	94	43.58	66.16

TESTS OF HOG SERUMS

Besides the testing of bovine serums, a limited number of tests were undertaken with hog serums. Most of these were obtained from abattoirs from hogs which on post-mortem examination revealed lesions of tuberculosis, irrespective of the extent of the disease. A desirable number of apparently healthy hogs were also included in the test. No detailed post-mortem charts accompanied the serums, they being only marked "positive" or "negative." A limited number of serums were also obtained from hogs which reacted to the intradermal tuberculin test and which on post-mortem examination proved to be affected with tuberculosis. In all, 120 hogs were tested.

The results of the complement-fixation test in tuberculous hogs were very irregular and much less reliable than in cattle. This might be attributed to the fact that tuberculosis in hogs as observed in meat inspection is limited in about 95 per cent of the infections to the lymph glands of the head and neck in the form of isolated foci, showing in most

instances a retrogressive tendency. Such moderate lesions might not induce the formation of sufficient antibodies in the animal to permit detection by the test.

TESTS OF HUMAN SERUMS

A limited number of human serums were also included in the tests in order to establish whether the results with a certain antigen would be more reliable with animal serum than with human serum. In all, 22 cases were tested. Most of the positive serums were obtained from patients of the Tuberculosis Hospital at Washington, D. C., through the courtesy of the authorities. These tests are summarized in Table X.

TABLE X.—*Summary of the tests on human serums conducted with an antigen prepared from a bacillary emulsion and tuberculin precipitate*

Human serum	Number of cases	Degree of fixation						Percentage.
		++++	+++	++	+	±	−	
1 Apparently healthy	5						5	100
2 Clinical symptoms present, sputum negative	4				1	2	1	33
3. Clinical symptoms moderate, sputum positive	3	2					1	67
4 Clinical symptoms progressive, sputum positive	6	3	1	1	1			100
5 Clinical symptoms severe (cavities), sputum positive	4		1	1			2	50
Total	22	5	2	2	2	2	7	70

From Table X it appears evident that the results are not more reliable than in bovine tuberculosis and also that the degree of the reaction has no bearing on the extent of the disease. However, it is recognized that the number of human cases tested does not warrant any definite conclusions.

CONCLUSIONS

(1) The complement-fixation test for the diagnosis of tuberculosis in cattle is not so reliable as the subcutaneous tuberculin test.

(2) Since a large proportion of the positive cases give only a faint reaction, it necessitates a very careful titration of the antigen and a most accurate observance of all of the details of the technique of the test.

(3) The amount of fixation can not be considered as an index to the extent of infection. Frequently samples from animals showing arrested, retrogressive lesions gave a more marked fixation than animals affected with acute, progressive, generalized tuberculosis.

(4) The complement-fixation test might be employed as a supplementary test in cases of doubtful or atypical reactions to the subcutaneous or other allergic tests. It is not practical for general diagnostic purposes.

(5) The subcutaneous tuberculinization of healthy animals affects the results of the complement-fixation test. Such interference may be noted as early as on the fourth day following the injection and may persist for at least six weeks, and possibly for a much longer time.

(6) There is no material difference in the reliability of the test in cattle as compared with human beings.

(7) Comparative tests with various antigens proved that one prepared with bacillary emulsion and tuberculin precipitate was the most effective.

(8) There appears to be no constancy in the presence of antibodies in tuberculous animals.

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AVAILABILITY OF POTASH IN CERTAIN ORTHOCLASE-BEARING SOILS AS AFFECTED BY LIME OR GYPSUM

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INTRODUCTION

Most soils appear to contain sufficient calcium to supply an abundance of that element so far as the actual food requirements of the crop are concerned. In the majority of cases in agricultural practice the application of lime, either as quicklime (CaO) or as the carbonate ($CaCO_3$), is made either on account of its effect upon the physical condition of the soil or to neutralize soil acids. There are cases, however, where a relatively small application of lime will accomplish beneficial effects that are not readily explainable on the basis of either of these postulates. The same is true with calcium sulphate, or gypsum ($CaSO_4.2H_2O$).

Such instances have perhaps helped to formulate the idea, apparently quite widely held, that calcium replaces potassium in the potash-bearing minerals of the soil, liberating this plant-food element. Thus, Hilgard,[1] in summarizing the important chemical effects of calcium carbonate, includes among others "the rendering available, directly or indirectly, of relatively small percentages of plant food, notably phosphoric acid and potash"; and again, in speaking of calcium sulphate, Hilgard makes the following statement:

> Being soluble in 400 parts of water, it easily penetrates downward in most soils, and in doing so effects changes in the zeolitic portions, setting free potash from silicates and thus indirectly supplying plants with this essential ingredient in a soluble form. About 200 pounds per acre is an ordinary dose.[2]

Lyon, Fippin, and Buckman speak of the action of gypsum in liberating potassium, as "a property with which it has generally been credited" . . . "the actual extent of which has never been very clearly demonstrated."[3]

André[4] reports a greatly increased solubility of the potash in microcline in the presence of calcium carbonate or calcium sulphate, the solubility being 2 and 2.5 times, respectively, that in water alone.

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Such reactions as may exist can not be considered to be independent of the character of the potash-bearing minerals in the soil. Prianischnikow [1] has shown with a number of different crops that marked differences exist in the availability of the potash in the various potash minerals, and that the availability corresponds approximately with the solubility of the potash of the different minerals in solutions of neutral salts. Of all the potash-bearing minerals tested, those of the feldspar group (orthoclase, microcline) showed the lowest available potash.

Blanck [2] has found that the potash in biotite and muscovite, minerals of the mica group, is more available to plants than that in orthoclase. The addition of finely ground microcline and orthoclase (equivalent to 1.6 gm. of potassium oxid) to sand (18 kgm.) having a very low potash content, resulted in little or no increase in the dry weight of oat plants, compared with plants grown in sand alone, and did not appreciably increase the potash content. An equal amount of potassium oxid applied as potassium sulphate increased the yield 70 per cent. Blanck concludes that the availability of the potash in potash-bearing minerals to plants increases proportionally to their sodium and calcium content.[3]

Many of the soils devoted to the culture of Citrus fruits in southern California have been derived from granite, pegmatite, or other rocks of this general type. Under the prevailing arid conditions the decomposition of the bedrock has been slow, and small lumps of granite, feldspar, and silica can still be found scattered plentifully through the soil.

Pegmatite and orthoclase were selected as types of the potash-bearing rocks and minerals that constitute the chief source of potassium in many of these Citrus soils. Samples collected near Riverside, Cal., were ground and passed through a 60-mesh sieve. Upon analysis by fusion, the pegmatite gave 1.50 per cent of potassium oxid and the orthoclase 12.56 per cent. The following experiments indicate that the addition of lime or gypsum to soils derived from orthoclase-bearing rocks has little or no effect, so far as the liberation of potash is concerned.

SOLUBILITY OF THE POTASSIUM IN PEGMATITE AS AFFECTED BY CALCIUM HYDRATE AND CALCIUM SULPHATE

Ten-gm. portions of pegmatite were weighed out and placed in shaker bottles with 1 liter of water, together with varying amounts of calcium hydrate (expressed as calcium oxid), as given in Table I. These mixtures

[1] Prianischnikow, D. Vegetationsversuche mit verschiedenen kalihaltigen Mineralien. *In* Landw. Vers. Stat., Bd 77, Heft 5/6, p. 399-411, pl 2-6. 1912.

[2] Blanck, E. Die Bedeutung des Kalis in den Feldspaten für die Pflanzen. *In* Jour Landw., Bd. 61, Heft 1, p. 1-10. 1913.

[3] The solubility of the feldspar at temperatures above that of the soil is manifestly beyond the scope of this paper. Mention may appropriately be made, however, of a recent investigation by Stephenson, who has compared the action of various alkaline solutions on feldspars and hornblende at 100° C. and above. (Stephenson, E. A. Studies in hydrothermal alteration. Pt. I. The action of certain alkaline solutions on feldspars and hornblende. *In* Jour. Geol., v. 24, no. 12, p. 180-199, 8 fig. 1916.)

were shaken for 13 days and the clear solutions then analyzed for potash by the colorimetric method described by Cameron and Failyer.[1] The amounts of potash in these solutions is shown in Table I.

TABLE I.—*Effect of solutions of calcium hydroxid on the solubility of the potassium in pegmatite*

Solution No.	Calcium oxid in 100 c. c. of solution.	Potassium oxid in solution.
	Gm.	*P. p. m.*
1	0. 00	3 1
2	. 0123	3. 1
3	. 0246	2. 5
4	. 0369	3. 1
5	. 0492	3 0
6	. 0738	3 0
7	. 0984	3. 1
8	[a]. 1230	2 8

[a] Solid phase present.

The amount of potash recovered is so nearly uniform that these results could very well be considered to be multiplicate determinations of the potash content of the same solution. There is no evidence that the lime liberated any potash from pegmatite.

Other 10-gm. portions of pegmatite were weighed out and placed in shaker bottles. To each portion was added 1 liter of distilled water, with varying amounts of calcium sulphate, as shown in Table II. These mixtures were shaken for six days and the solutions then analyzed for potash. The amounts of potash found in solution are given in Table II.

TABLE II.—*Effect of solutions of calcium sulphate on the solubility of potash in pegmatite*

Solution No.	Calcium sulphate in 100 c. c. of solution	Potassium oxid in solution
	Gm	*P p m*
1	0. 00	2. 7
2	. 0221	2. 1
3	. 0446	2. 1
4	0668	2. 0
5	. 0864	3. 2
6	. 1330	2. 2
7	. 1660	2. 1
8	[a]. 2100	2. 8

[a] Solid phase present.

As in the case of the calcium-hydrate solutions, there is no indication that the presence of calcium sulphate led to any replacement of the potash in pegmatite by lime.

[1] Cameron, F. K., and Failyer, G. H. The determination of small amounts of potassium in aqueous solutions. *In* Jour. Amer. Chem. Soc., v. 25, no. 10, p. 1063–1073 1903.

SOLUBILITY OF THE POTASSIUM IN ORTHOCLASE AS AFFECTED BY CALCIUM HYDRATE AND CALCIUM SULPHATE

Measurements relating to the effect of calcium hydrate on the solubility of potash in orthoclase, carried out in a similar manner to those with pegmatite, are given in Table III. Here, again, there is no indication that the presence of calcium affects the solubility of the potash.

TABLE III.—*Effect of solutions of calcium hydrate on the solubility of the potash in orthoclase*

Solution No	Calcium oxid in 100 c. c. of solution	Potassium oxid in solution.
	Gm	*P. p. m.*
1.	0.00	10.8
2.	.0103	8.6
3	.0207	12.0
4	.0414	8.2
5	.0621	9.1
6	.0828	12.6
7	.1035	12.1
8	[a] .1242	9.6

[a] Solid phase present.

The results of similar determinations of the solubility of the potassium in orthoclase in the presence of solutions of calcium sulphate of varying concentration are presented in Table IV. A different sample of orthoclase was used in this case from that employed in the calcium hydroxid series, which accounts for the difference in solubility of the feldspar in distilled water in the two series. As before, there is no indication that potash is being replaced by lime. On the other hand, the results indicate that increasing the concentration of the calcium sulphate depresses the solubility of the potash.

TABLE IV.—*Effect of solutions of calcium sulphate on the solubility of the potash in orthoclase*

Solution No	Calcium sulphate in 100 c. c. of solution	Potassium oxid in solution.
1	0.00	6.0
2	.018	4.5
3	.035	3.6
4	.070	3.6
5	.105	1.2
6	.140	.8
7	.175	1.3
8	[a] .210	.5

[a] Solid phase present

SOLUBILITY OF THE POTASH IN SOILS OF THE RIVERSIDE AREA AS INFLUENCED BY CALCIUM HYDRATE AND CALCIUM SULPHATE

A large sample of virgin soil from the site of the new University of California Citrus Experiment Station at Riverside was collected, dried in the sun, and passed through a 1-mm. sieve. Portions of this soil of 100 gm. each were weighed out and 500 c. c. of distilled water added, together with varying amounts of calcium hydroxid, as shown in Table V. The mixtures were shaken until the solutions had reached equilibrium. The amounts of potash found in the solutions are given in Table V As in the case of the pegmatite and orthoclase experiments, the addition of calcium hydroxid does not modify the solubility of the potash in this soil sufficiently to be detected in the analyses.

TABLE V.—*Effect of solutions of calcium hydrate on the solubility of the potash in soil from the Citrus Experiment Station site*

Solution No	Calcium oxid in 100 c. c. of solution	Potassium oxid in solution
	Gm	*P. p. m.*
1	0.00	26.4
2	.0103	26.4
3	.0207	28.8
4	.0414	28.8
5	.0621	24.0
6	.0828	27.6
7	.1035	25.2
8	[a] .1242	27.6

[a] Solid phase present

The solubility of the potash in the soil from the Citrus Experiment Station site in the presence of varying amounts of calcium sulphate was also determined. The concentration of the calcium-sulphate solutions and the amount of potash found in solution are given in Table VI

TABLE VI.—*Effects of solutions of calcium sulphate on the solubility of the potash in soil from the Citrus Experiment Station site*

Solution No	Calcium sulphate in 100 c. c. of solution	Potassium oxid in solution
	Gm	*P. p. m.*
1	0.00	24
2	.017	24
3	.034	26
4	.068	29
5	.102	26
6	.136	26
7	.170	29
8	[a] .210	26

[a] Solid phase present.

The average variation (1 part in 25) in the potash content is well within the experimental error, when the lack of uniformity in the soil and the analytical difficulties are considered. There is no evidence that the addition of calcium sulphate modified to a measurable degree the solubility of the potash in this soil.

Another soil from the Oatman tract, about 7 miles from Riverside, was treated with calcium-sulphate solutions in the same way. This soil, which had been under cultivation for a number of years, was more granitic in character and less weathered than the virgin soil. The potash solubility in varying concentrations of calcium sulphate is shown in Table VII. Here, again, there is no indication of any replacement of potash by the lime; but, on the contrary, the addition of calcium sulphate depresses the solubility of the potash, as was found in the case of orthoclase (Table IV).

TABLE VII.—*Effect of solutions of calcium sulphate on the solubility of potash in Oatman soil*

Solution No.	Calcium sulphate in 100 c. c. of solution	Potassium oxid in solution.
	Gm.	*P. p. m.*
1	0.00	8.6
2	.017	8.6
3	.034	8.0
4	.068	4.2
5	.102	2.2
6	.136	2.2
7	.170	2.4
8	.210	4.2

[a] Solid phase present.

LeClerc and Breazeale[1] have shown that the sprouting seedling of wheat manifests a strong demand for potash, entirely out of proportion to its demand for nitrogen and phosphoric acid. When potash is present in a readily available form, the young wheat seedlings, during the first three weeks of growth, will often take up an amount of potash (K_2O) equivalent to 8 per cent or more of their dry weight. This avidity of the plant for potash was made use of in the following manner: Wheat seeds were sprouted on perforated aluminum disks floating in large pans. Each pan contained about 1,000 seeds. When the shoots had reached a length of about 2 cm., the plants were taken up, a few at a time, and transplanted to other disks, floating in the various solutions listed in Table VIII. In this way all the unsprouted seeds were eliminated, which would otherwise have become a source of potash. The solutions in the pans were stirred frequently and the plants were

[1] LeClerc, J. A., and Breazeale, J. F. Translocation of plant food and elaboration of organic plant material in wheat seedlings. U. S. Dept. Agr. Bur. Chem., Bul. 138, 32 p., 2 fig. 1911.

allowed to grow for varying periods, ranging from 5 to 12 days. Plant samples were then withdrawn and analyzed for potash by the official method. Table VIII shows the treatment and the percentage of potassium oxid in each lot of plants, expressed in terms of the dry weight.

TABLE VIII.—*Potash absorbed by wheat seedlings from orthoclase and soil solutions as affected by calcium sulphate.*

Series No	Treatment	Percentage of potassium oxid in dry plants
1	10 gm. of orthoclase, 2,500 c. c. of carbon-treated water	1.10
1	Same, saturated with calcium sulphate	.95
2	10 gm. of orthoclase, 2,500 c c. of carbon-treated water	1 84
2	Same, saturated with calcium sulphate	1.72
3	40 gm. of orthoclase, 2,400 c. c. of carbon-treated water plus 200 p. p. m. NO_3 and 200 p. p. m. P_2O_5	2 56
3	Same, saturated with calcium sulphate	2. 57
4	50 gm. of Oatman soil, 2,500 c c of carbon-treated water	1 75
4	Same, saturated with calcium sulphate	1.35

The period of growth was varied so that the dry weight of the plants in the different series was not the same, which accounts for the differences in the potash content of the various cultures when expressed in percentage of the dry weight. In the third series 200 p. p. m. of NO_3 as $NaNO_3$ and 200 p. p. m. of P_2O_5 as Na_2HPO_4 were added to balance the nutrient solution.

To insure the solution of the potash, the solutions containing finely ground orthoclase in series 3 were shaken up for four days before they were used for culture purposes. Analyses of the resulting solutions at the time the cultures were started showed 9.6 p. p. m. of potassium oxid where gypsum was absent and 6 p. p. m. where gypsum was present. Such potash concentrations are sufficient for the nutrition of plants, as the potash content of the soil solution in humid soils seldom exceeds 10 p. p. m. It will be shown in another paper that wheat plants can readily take up potash and other salts in concentrations of 1 p. p. m. or less

If the calcium sulphate had liberated any additional potash from the feldspar, the seedling plants would have taken it up, and this would have been shown by an increase of the potash content of the plant ash. In none of the three series of cultures, however, was the potash content of the plants increased by the addition of gypsum to the solution.

In the Oatman soil (series 4) the addition of gypsum decreased the absorption of potash by plants. It will be recalled that the analyses of the solutions also showed that the use of gypsum depressed the solubility of the potash in this soil (Table VII).

SUMMARY

It is stated in agricultural treatises that the application of lime to a soil liberates potash from the soil minerals. This subject is of special import to the Citrus industry of southern California in which commercial fertilizers are extensively used and heavy applications of lime and gypsum are sometimes made.

Samples of pegmatite and orthoclase were collected near Riverside, Cal., representing, respectively, types of the potash-bearing rock and mineral from which many of the Citrus soils appear to be derived. These samples were finely ground and shaken for a number of days with aqueous solutions of calcium hydroxid and of calcium sulphate in graduated concentrations. The calcium-hydrate solutions did not modify the solubility of the potassium in either pegmatite or orthoclase. Gypsum solutions depressed the solubility of the potassium in orthoclase, the quantity of potash in solution decreasing progressively as the concentration of the calcium sulphate increased.

Similar tests were made upon a virgin soil of a granitic type from the experiment station near Riverside, Cal. The solubility of the potash was not measurably different in distilled water and in solutions of calcium hydrate or calcium sulphate.

The addition of calcium sulphate to a Citrus soil which had been under cultivation for some time and which was more granular and less weathered than the virgin soil, decreased the solubility of the potash.

The potassium content of wheat seedlings was practically the same when grown (1) in water containing finely ground orthoclase and (2) in a saturated calcium-sulphate solution containing the same quantity of orthoclase.

Similar experiments in which a Citrus soil was used instead of orthoclase showed a decreased absorption of potassium by wheat seedlings in the presence of calcium sulphate.

In brief, the experiments indicate that the availability to plants of the potash in soils derived from orthoclase-bearing rocks is not increased by the addition of lime or gypsum. In some instances a marked depression of the solubility of the potash in the presence of gypsum was observed. These conclusions are based both on the results of the analyses of the solutions and on the measurement of the potash content of wheat seedlings grown in the solutions.

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EWES' MILK: ITS FAT CONTENT AND RELATION TO THE GROWTH OF LAMBS

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Incidental to the major lines of research in the sheep-breeding experiments carried on at this Station some observations were made on comparative milk yields and tests of their fat content. The data on this subject cover 6 distinct breeds [1] and 11 types of first-generation crosses of sheep which may properly be considered as nonmilk breeds. It is obvious that such data can not be obtained with a degree of reliability equal to that possible in animals kept for dairy purposes. While the data shown here can not be regarded as final, yet it is the belief that it does in the main express the existing state of conditions. It is therefore presented as a contribution to a subject on which information is very meager in the hope that it will attract attention to the importance of the milk-yielding characteristics of our early-maturing mutton breeds of sheep. The fact that the sale of good, early lamb is becoming one of the prime requisites of profit in sheep husbandry under average farm conditions emphasizes the necessity of selecting breeding ewes with some regard to their potentialities as milkers.

While the fat content of ewes' milk has not been the subject of much research, the data available on the subject indicate that ewes' milk has a much higher average of fat than cows' milk. One of the interesting features of ewes' milk suggested by these tests, however, is the great variation that exists between individuals of a breed and the variation in the milk of single individuals at different periods during lactation or during different lactation periods.

There exists apparently very little difference in this respect between breeds which have been selected for large milk yields and those that are not bred especially for milk, including the more common English breeds and those of the Merino type. Of the former, Hucho (6) [2] gives the analyses of milk from three East Friesian ewes, showing the ranges, respectively, of 4.32 to 10.80, 4.35 to 7.50, and 4.15 to 7.38. Besana (1) gives 9.50 as the average test of 176 samples from an Italian breed, the samples representing a period 21 days after lambing.

[1] Native sheep are here considered as a distinct breed.

[2] Reference is made by number to "Literature cited," p. 35-36.

Trillat and Forestier (14) report 6.98 as the average of 10 samples from ewes of the Roquefort region of France, and Sanna (13) gives 7.53 as the average of 55 samples from sheep in southern Sardinia.

For the nonmilk breeds Fuller and Kleinheinz (5), of the Wisconsin Station, give the following analyses: Oxford, 7.65; Southdown, 8.4; Dorset, 7.2; Shropshire, 5.88; Merino, 6.00; and Montana, 7.15. These figures represent the averages of several individuals of each breed, which compare very closely with averages obtained at this station.

Tables I and II give the analyses of 158 samples of ewes' milk. They represent 95 ewes of 6 distinct breeds and 11 crosses, the individual animals ranging in age from 1 to 8 years. Each sample represents a composite of three or more milkings, taken on an equal number of days from one nipple, which was milked dry, the lambs being kept apart from ewes from 11 a. m. to 1 p. m., when the samples were drawn. All samples were taken approximately one month after lambing, which represents the period of lactation when the maximum yield would be expected under normal conditions.

TABLE I.—*Average and range of fat content of ewes' milk by breeds at different ages*

Breeds and crosses.	Number of samples.	Range.	Average.	2 years		3 years		4 years.	
				Samples.	Fat.	Samples.	Fat.	Samples.	Fat.
		Per cent	*P. ct.*		*P. ct.*		*P. ct.*		*P. ct.*
Native	3	6.2–10.5	7.9						
Shropshire	9	3.8–10.0	6.8	3	7.1	3	8.3	1	3.8
Dorset	13	3.8–12.1	6.0	5	5.4	3	6.8	1	12.1
Southdown	11	4.2–10.7	7.6	4	6.9			1	5.1
Hampshire	11	2.4–7.4	4.7	3	3.4	1	2.4	2	10.7
Rambouillet	20	3.3–9.7	6.0	1	8.0	3	5.9	6	6.3
Dorset×Rambouillet	9	3.5–7.1	5.1	4	4.1	4	5.7	1	7.1
Southdown×Rambouillet	4	4.9–6.5	5.8	2	5.5	1	5.6		
Shropshire×Rambouillet	13	3.8–10.4	7.0	5	6.1	7	7.1	1	10.4
Hampshire×Rambouillet	29	2.4–8.6	5.4	10	6.0	8	5.0	3	6.5
Hampshire×Rambouillet F_2	1		8.4	1	8.4				
Dorset×Southdown	4	4.3–7.6	5.6	2	6.9	1	4.6	1	4.3
Southdown×Dorset	3	7.2–9.0	7.9	2	8.3	1	7.2		
Southdown×Native	2	6.2	6.2	1	6.2			1	6.2
Dorset×Native	1		5.6					1	5.6
Shropshire×Native	13	3.6–11.4	6.1	7	5.6	5	7.2	1	1.6
Hampshire×Native	12	3.4–8.0	5.4	5	5.3	5	5.8	2	4.7
Average, all breeds	158		6.0	55	5.8	42	6.2	22	6.2
Range, all individuals.	158	2.4–12.1		2.7–9.5		2.4–11.4		3.5–12.1	

Breeds and crosses.	5 years.		6 years.		7 years.		8 years.	
	Samples.	Fat.	Samples.	Fat.	Samples.	Fat.	Samples.	Fat.
		P. ct.		*P. ct.*		*P. ct.*		*P. ct.*
Native	2	8.4	1	7.1				
Shropshire	1	4.0	1	7.5				
Dorset			3	4.8	1	4.0		
Southdown	2	7.7	2	9.0	1	7.0	1	10.7
Hampshire	3	5.3	2	4.8				
Rambouillet	4	6.9	5	5.0	1	3.6		
Hampshire×Rambouillet	3	4.2	3	4.8	2	5.9		
Average, all breeds	15	6.38	17	5.6	5	5.3	1	10.7
Range, all individuals	2.4–10.5		3.0–9.8		3.6–7.0			

A study of Table I shows an interesting condition as regards comparisons between breeds. The marked feature is the tendency toward variation within breeds rather than between breeds. This is especially evident in column 2, which shows the range within breeds. In view of this condition, the variations between the breed averages shown in column 3 lose most of their significance on account of the small numbers available.

In the totals for all breeds the averages and range for different age periods have been shown. These averages are practically equal for any age period from 2 to 7 years. The ranges also at different periods of age shown in this summary are approximately equal among ewes at any period from 2 to 7 years. The suggestions derived from this table are strictly in line with those obtained from Table II—that fat is a factor which is exceedingly variable in individual ewes regardless of breed or age.

Table II shows ewes that have been tested for two or more years. Column 1 shows the number of the animal; columns 2, 3, and 4 the fat test of milk in a similar number of lactation periods or successive years.

TABLE II.—*Variation in the fat of the milk of individual ewes in different years*

Animal No	1st test	2d test	3d test	Animal No	1st test	2d test	3d test
	Per cent	*Per cent*	*Per cent*		*Per cent*	*Per cent*	*Per cent*
79	3 8	7 5		126	3 4	4 8	4 0
149	7.1	10 0		127	5 1	4 6	5 3
150	6 4	8.4		221	6 3	7 1	
205	7.8	6 4		223	3 6	6 8	
9	4.0	4.0		224	8 0	5 5	
158	4 4	11 4		83	2 4	5 8	4 8
159	4.4	5 2		84	5 0	5.6	7 0
160	6 4	3 8		85	5 6	5 2	3 0
16	5.1	6 8	8 2	113	6 3	6 4	
24	9 8	7.0		115	3	5 2	7 4
74	7 4	6 3		211	6 1	4 7	
76	4 5	6 8	3 2	214	2	4 1	
110	2 7	2.4		215	5 7	4 0	
40	6 3	5.2		218	8.6	6 1	
41	7 8	3 5	7 4	219	5.8	4 9	
143	3 5	5 8	7 1	120	3 3	4 8	
162	4 6	6 6		189	6 5	5 2	
132	6.1	4.6	4.3	194	9.0	5 8	
176	9 0	7.2		105	5 7	6 2	
103	6 2	6 2		106	4 8	7 5	3.0
284	6 5	4 9		197	3 8	11 4	
146	4 6	7 3	10 4	200	7 8	5 3	
147	3.8	7 0					
201	5.6	5.7		Average	5 67	6 03	5.81

The outstanding feature of Table II is the remarkable variation obtained in the fat content of individual ewes tested in different lactation periods.[1] This indicates that no great reliance can be placed on single tests of an individual, and that a test must either cover a larger number of periods during one lactation of an individual or that it must cover an average of a large number of individuals at one period, in order to be representative. This latter condition, which is shown in the aver-

[1] Lack of experimental evidence leaves it open to question whether an equal lack of uniformity would be obtained in a number of tests made during the same lactation period of an individual

age for all ewes, 46 in number, gives practically similar results for three successive lactation periods.

Fat is still quantitatively the most variable factor of the solids in milk from breeds of animals in which selection has been practiced for generations to increase the fat content. Such selection has so far failed to stabilize the percentage of fat in milk partly because no definite limits have been set to the standard.

If selection without a definite limited standard as an objective results in instability, a possible inference would be that the milk of animals in which no selection is made in either the quantitative or qualitative factors would become more stable in this respect. The literature cited on this point is corroborated by the data shown in this paper that this is not the case. In our mutton breeds of sheep, in which no endeavor has been made to modify the fat content or increase the milk yield by selection, there exists apparently an equally unstable variation as regards the percentage of fat in milk.

In breeds of animals that are kept for dairy purposes this variability is the factor of basic importance. In breeds of sheep that are not kept for dairy purposes and where the entire yield is suckled by their young, a variation in fat content of milk can assume importance only inasmuch as it may be a limiting factor in the rapid growth of suckling lambs. This question is partly answered in Table III, which shows increases in the weights of lambs from milk varying in fat from 2 to 10 per cent. The increase shown from the milk richest in fat is no greater than that from the milk which has the lowest fat content. In fact, the summary shows the highest gains (37 pounds) from 2 to 3 per cent milk, and the lowest gains (18 pounds) from milk testing 10 per cent or over. The limiting factor in this case was apparently the quantity of milk, as the high gains were made from good to high milk yield and the low gains from poor to good milk yield. On the elimination of these extreme cases a considerable fluctuation in weight increase is found that bears no definite relationship to the percentage of fat contained in the milk within the limits shown here.

TABLE III.—*Average increase of lambs in weight at 8 weeks on varying quantities of dams' milk varying in fat*

Number of ewes	Milk yield (estimated)	Average fat test	Weight increase.									
	Fat content per cent		2 to 3	3 to 4	4 to 5	5 to 6	6 to 7	7 to 8	8 to 9	9 to 10	10 or over.	Average.
		Per ct	*Lbs.*	*Lbs.*	*Lbs*	*Lbs.*	*Lbs*	*Lbs*	*Lbs.*	*Lbs.*	*Lbs.*	*Lbs.*
13	High.....	4 82	42.0	35.0	29.0	38 2	34.0	42.0	29.0		.. . .	34.0
78	Good..... ...	6. 15	32 5	31 0	36.0	31 0	32.0	33 0	31.0	25.0	25 0	29.3
35	Fair..	6.05	.	25.0	22 5	27 5	26. 5	24.0	26.0	23.0	22 0	24.6
12	Poor	6 03		15.0	21.0	19 0	26.0	19.0	22.0	..	9 0	19.0
138	Average... .		37 0	26 5	27 1	30.0	29.6	29.5	27.0	24.0	18.7	....

Weight increase in the development of an early lamb from birth to the time when it is most profitably marketed (approximately three months) is marked by two distinct processes of increment: (1) Growth [1] and (2) mast, or fattening. It is obvious that the first is the more important process, since it determines size and weight. Fattening is merely a padding of the structural tissues of the body and does not contribute material for growth or structural increase. It is obvious that fattening would be of little value in young lambs which failed to make growth.[2] On the other hand, young animals may make good growth and yet not present that condition of "finish" demanded by the market if deposition of fat has not gone hand in hand with growth, a condition not uncommonly found in calves fed on skim milk.

The direct body-increment value of milk fat then lies chiefly in its capacity to promote mast simultaneously with growth, so far as the growing of early lambs for market is concerned. That milk fat is necessary and can not be entirely substituted with success in the ration of growing animals is conclusively shown by the experiments of Mendel and Osborne (11).[3] Their observations are in entire accord with the practical knowledge of feeders and breeders that young animals make a more rapid continued growth when given whole milk. This is well illustrated by the fact that no successful exhibitor would fit calves for the show ring with skim milk. Milk fat here is shown to have an indirect effect on weight increase by stimulating growth. Since fat in this capacity serves as a stimulus and not as a material for structural increase (growth), its quantitative variability in milk is probably not so important from this standpoint, a conclusion which is also suggested by the practical results shown in this paper. In other words, milk which is normally low in fat appears still to supply a sufficient amount of this substance to growing animals, provided it is available in sufficient quantity to meet the demand for protein and ash—that is to say, the demand for proteins and ash is relatively so much greater than the demand for fat in growing animals of suckling age that milk which is quantitatively sufficient to supply the former will contain a large enough total to answer all demands for the latter.

RELATION OF MILK YIELD TO GROWTH OF LAMBS

The effect of the quantitative factor in the milk yield of ewes on successful sheep raising under our ordinary farm conditions is a question of economic importance, although little or no attention is being paid to it

[1] Repair and maintenance are here included with demands for growth

[2] Just the contrary is true in mature animals that have finished their growth.

[3] "It has been shown that this cessation of growth can be stopped and growth resumed by the substitution of other naturally occurring fats for part of the lard in the ration. In butter fat, egg fat, cod-liver fat, and beef fat, and more specifically in the fractions of these containing the oil components liquid at ordinary temperatures, there exists a determinant of growth in the sense in which this expression has been discussed above." (11, p. 216.)

by breeders. Some indication of this is given in Table III. It is obviously difficult to determine the absolute yield in animals that are not milked regularly by hand. The classification shown here is therefore based on a relative comparison rather than on absolute yield. This classification was based on close observation and verified by the actual quantities drawn for the determination of fat. Four grades were established to indicate relative yield: (1) High, (2) good, (3) fair, and (4) poor. The results shown in the last column of Table III and also by the curves in figure 1 bring out clearly the economic significance of high yield. The number of ewes represented is fairly large and the results should there-

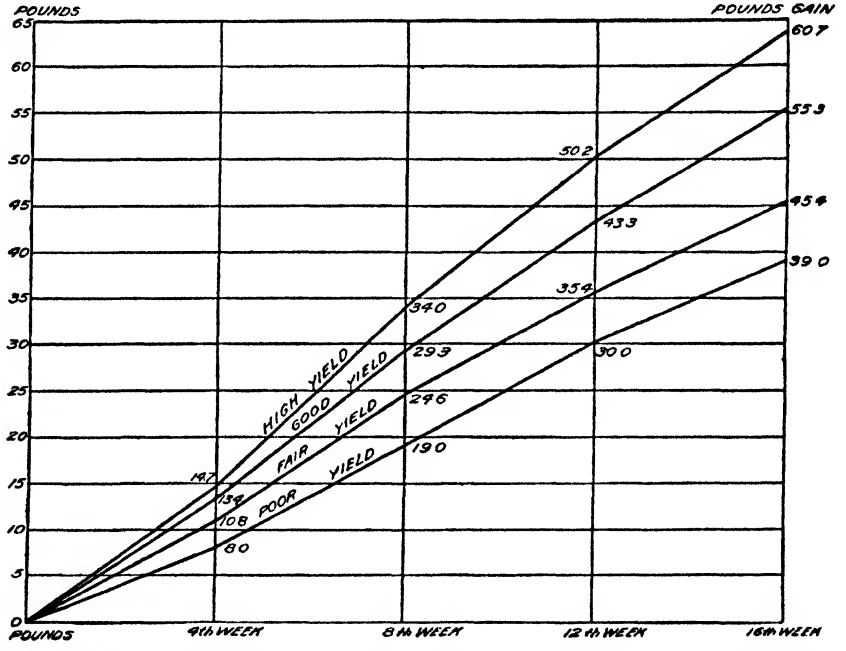

FIG. 1.—Curves showing rate of increase in weight of lambs (in pounds) by 4-week periods from different quantities of ewes' milk

fore possess a fairly representative value as to facts. The difference in weight increase between lambs from high-milking ewes and good-milking ewes is 16 per cent; between high- and fair- milking ewes, 38 per cent; and between high- and poor- milking ewes the difference is 79 per cent. The same results from different yields of milk are also represented graphically in figure 1. The curves here show a rise almost directly proportional to yield. As shown in 4-week periods, the greatest rate of increase is found in the second period for all groups. A slight decline in the rate of growth is found in the third period, except in the low yield, and is followed by a still further decline in the fourth period. Since all lambs had free access to a liberal amount of grain and hay, which they ate greedily as they

grew older (third and fourth periods) the controlling factor must have been the milk. The marked feature of figure 1 is the uniformity in the rate of growth between the different groups modified in degree by the relative milk yield of the dams. Such results hardly need further comment to emphasize their significance.

While some of this weight increase represents mast, due to the effect of milk fat, yet the limiting factor is the quantitative supply of material available for direct structural increase—namely, proteins and ash, the latter concerning chiefly lime.

Aside from the influence of inherited capacity, mast has limitations in promoting net increase dependent on rate of growth. Growth, on the other hand, is not dependent on mast. Its limitations are set mainly by inherited capacity and an abundance of proper food. In other words, weight increase from mast can be only relative, whereas growth is not limited in the same sense. Recent investigations into the nature of growth give added significance to the importance of an abundant supply of whole milk during the earlier stages of adolescense. These concern not only the specific functions of the various ash constituents (2, 3, 4, 7, 8, 9) in metabolism but also the newer interpretations of the structural differentiation among the various amino-acid derivatives of protein and their respective effect on growth (10, 11, 12). Protein under these circumstances loses its generic value, and its character and source become a matter of as great importance as its quantitative sufficiency.

The function of nutrition is in a sense more complex in the growing animal than in the adult, since it involves more metabolic processes. The statement may seem somewhat paradoxical, in view of the well-known fact that the digestive organs of the young are comparatively rudimentary. Nature has overcome this difficulty in providing the proper food in milk. Although its virtues have always been recognized on the basis of practical results, yet the mere statement of fact that it is perfectly balanced and easily assimilated take on added meaning when viewed in the light of such results as those of the physiologists cited.

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IMMUNITY STUDIES ON ANTHRAX SERUM

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INTRODUCTION

Beginning with the classical work of Pasteur (9)[1] in 1881, wherein he conclusively showed that it was possible through the use of attenuated cultures of *Bacillus anthracis* to immunize animals against otherwise fatal doses of anthrax organisms, various investigators have from time to time taken up work along this line and through extensive experimentation have contributed much toward our knowledge of anthrax, its treatment, and methods of immunization against it.

While in general Pasteur's vaccine proved highly satisfactory and has been extensively used with excellent results, it has a number of disadvantages—that is, it requires two handlings of the animals; the desired degree of immunity is not reached until approximately a week or 10 days after the injection of the second vaccine; there is a small percentage of losses in vaccinated animals due directly to the vaccine; and the keeping qualities of the vaccine under unfavorable conditions are not the best (4). These factors led a number of investigators to attempt various modifications of Pasteur's method. It being possible to immunize susceptible animals against anthrax, some workers directed their efforts toward the preparation of an immune serum through hyperimmunization. Thus, in 1895 Marchoux (8), by immunizing sheep according to Pasteur's method and then hyperimmunizing them through injections of increasing doses of virulent anthrax culture, succeeded in preparing a serum capable of producing a passive immunity in susceptible animals. Further work along this line was conducted by Sclavo, Sobernheim, Mendez, Detre, Carini, and Ascoli. These investigators, however, employed larger animals, especially horses, and succeeded in producing potent immune serums. Serum of this type was found to be of considerable value in cases of threatening infection or where anthrax had already made its appearance in a herd, and in the treatment of the disease. The immunity conferred by anthrax serum, however, is of short duration, lasting only a few weeks. To produce a more lasting immunity Sobernheim (10) recommended a simultaneous treatment with serum and vaccine, the vaccine corresponding to Pasteur's second vaccine. Eichhorn (4) obtained good results from the use of serum and spore vaccine and found the method to possess advantages over the Pasteur method. Numerous experiments undertaken

[1] Reference is made by number to "Literature cited," p. 56.

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demonstrated the value of anthrax serum as a curative agent and as a prophylactic when employed simultaneously with anthrax spore vaccine.

Recalling the work on the separation of diphtheria antitoxin by fractioning the serum through the use of ammonium sulphate, the writers undertook the application of this method to anthrax serum and succeeded in producing the antibodies in a concentrated form. Chemical analyses of the serum and globulin preparations were made, and the changes in serum proteins during the course of hyperimmunization of animals against anthrax were studied.

SEPARATION OF PSEUDOGLOBULIN FROM IMMUNE SERUM

The method used was essentially similar to that described by Banzhaf (2) except that serum instead of citrated plasma was used. In all cases the serum was obtained from natural, spontaneously coagulated blood of two horses, which are here designated as horse 48 and horse 96. These had been hyperimmunized by the senior author, using an improved technic described in a previous publication (4). During the course of 6 months a total of 14 preparations of pseudoglobulin were made from 4 lots of serum from each horse. Serum 48 was known to have a high and serum 96 a comparatively low potency.

In the beginning it was not known whether the antibodies in the anthrax serum would withstand heating to 60° C., as in the diphtheria antitoxin preparation. For this reason each lot of serum was divided into two equal parts, one of which was given the heat treatment. Otherwise, the method of fractioning with ammonium sulphate, filtration, and dialysis was the same for both. The volume of serum used in each preparation varied from 600 to 1,200 c. c. (see Table I). Four preparations were simultaneously made—two from serum 48 (heated and not heated) and two from serum 96 (heated and not heated). This was done in order that the results of the subsequent inoculation experiments might be comparable. The serums from the two horses were not mixed, but were used separately in the pseudoglobulin preparation.

The serum was diluted with one-half its volume of water, and saturated ammonium-sulphate solution was added up to 30 per cent saturation—that is, 30 per cent of the total volume of the mixture. Thus, to 1 liter of serum there were added 500 c. c. of water and 643 c. c. of saturated ammonium-sulphate solution. Thirty per cent of the total volume, 2,143 c. c., consisted of saturated ammonium-sulphate solution, 643 c. c. At this concentration euglobulin was precipitated.[1] The mixtures to be heated were placed in an electrically heated drying oven maintained at 60° C. They were contained in 2-liter Erlenmeyer flasks provided with rubber stoppers. With few exceptions they remained in the oven for six hours and were then filtered along with the corresponding mixtures

[1] In diphtheria antitoxin preparation the euglobulin carries down little, if any, of the antitoxin. Banzhaf (2, p. 115).

that had not been heated. Hard papers were used (S. & S. 575, 24 cm. diameter) in glass funnels. The filtration was fairly rapid, not troublesome, and was allowed to go on overnight. The precipitated euglobulin was rejected. The filtrate contained the anthrax-immune bodies, pseudoglobulin and albumin.

The ammonium-sulphate content of the filtrates was now raised to 50 per cent saturation—that is, sufficient was added so that one-half of the final volume of the mixture consisted of saturated ammonium-sulphate solution. Thus, 1 liter of euglobulin filtrate contained 300 c. c. of saturated ammonium-sulphate solution, 233 c. c. of water, and 466 c. c. of serum. The addition of 400 c. c. of the saturated solution resulted in 1,400 c. c. of a mixture containing 700 c. c. of saturated ammonium-sulphate solutions, 233 c. c. of water, and 466 c. c. of serum. Such a mixture is "50 per cent" saturated. At this concentration pseudoglobulin was precipitated, carrying with it the anthrax-immune bodies. The mixtures were filtered as before, on hard papers. The filtrates containing albumin were rejected.

The precipitated pseudoglobulin was then freed from most of the adherent liquid by pressure between filter papers and towels, the precipitate being retained in the filter paper opened to form a semicircle. A convenient arrangement was the following: A towel; on top of this a large ordinary filter paper or two; on this, two filter papers containing precipitates. These two were covered with two ordinary filter papers and a towel, and so on. The pressure at first must be very slight and the filter papers and towels frequently renewed. After two or three days of pressure with gradually increased weights the precipitates were transferred to a press which pressed them into a condition resembling soft cheese. The color was white or nearly white. The pressed precipitates were easily removed from the papers with a spatula, were weighed, and transferred to parchment dialyzing bags. The weights varied, of course, with the quantity of serum used and other factors. From a liter of serum about 125 gm of moist, pressed pseudoglobulin were obtained. From 1 to 3 c. c. of chloroform were added, depending upon the amount of precipitate, and the bag was tied. The pseudoglobulin was dialyzed for 3 to 4 days against running tap water until only small amounts of sulphate were present in the tap water. The under surface of the bags was submersed in the tap water to a depth of 1 or 2 cm.

At the end of the dialyzing period the bags were opened and the volume of the globulin concentrate was measured. In all cases the reaction to litmus-paper strips was either neutral or faintly amphoteric; the odor of chloroform had disappeared, showing that the chloroform had dialyzed out and that there is no great danger of using too much chloroform in the beginning. The concentrates were odorless or nearly so. They were transferred to glass bottles and kept in a refrigerator. As a preservative 0.5 per cent chloroform was added. This has been found to be a most

suitable serum preservative by Voegtlin (11). The following quotation from his work (p. 118) is of interest:

> From the experiments described in this bulletin it would seem that chloroform when added to serum even to the point of saturation, is not capable of imparting to the serum a degree of toxicity which could be compared with that obtained on mixing serum with phenol and trikresol (0.25 and 0.5 per cent.). Practically the only effect which could be produced by the subdural injection of chloroform serum is the result of an increase in intracranial tension. With the use of the gravity method such results are not very apt to follow and it seems very doubtful that they occur at all. We, therefore, strongly suggest that chloroform be used as a preservative for antimeningitis serum. It is well recognized that serum preserved with chloroform after long standing will show a cloudiness which is probably due to the partial precipitation of the serum proteins. This fact, however, does not alter the efficiency of such a serum in the treatment of the disease.

With one or two exceptions the globulin concentrates were not Berkefeld-filtered. For analytic data on the serums and their concentrates see Table V.

Table I contains the data on the preparations of globulin concentrates from the serum. The figures in the last column are of particular interest. They show that on the average the concentrates from serum 48 contained 46 per cent, and from serum 96, 68 per cent, of the original total coagulable protein in the serum used in the preparations. In the inoculation tests serum 48 and globulin 48 were generally more potent than serum 96 and globulin 96.

TABLE I.—*Data on the preparations of globulin concentrates from serum*

Date when blood was drawn.	Volume of serum used for globulin concentration.	Heated at 60° C	Resultant volume of globulin concentrate.	Total coagulable protein in serum used.	Total coagulable protein in concentrate	Total protein in concentrate.	
SERUM 48							
	C. c.	*Hours.*	*C. c.*	*Gm*	*Gm.*	*Per cent.*	
July 26, 1915....	1,000	(a)	220	81.7	38.6	47	
Sept. 21, 1915..	650	0	190	49.8	27.0	54	
Do.....	650	6	115	49.8	15.7	32	
Nov. 5, 1915...	900	0	220	61.2	33.4	55	Average, 46
Do .. .	900	6	240	61.2	33.6	55	
Jan. 6, 1916. .	700	0	92	47.2	19.4	41	
Do	700	6	95	47.2	20.0	42	
SERUM 96							
July 26, 1915 . .	1,200	(a)	460	97.8	78.3	80	
Sept. 21, 1915....	650	0	255	55.9	36.8	66	
Do ..	650	3	235	55.9	33.2	59	
Nov. 5, 1915. .	900	0	404	79.8	59.5	74	Average, 68
Do	900	6	400	79.8	60.1	75	
Jan. 6, 1916 .. .	700	0	146	55.2	32.8	59	
Do	700	6	158	55.2	34.6	63	

[a] Heated in water bath at 60° C. until temperature inside of flasks was very near 60°.

Ascoli (1) separated the pseudoglobulin from anthrax serum and showed that the immune bodies were contained in this fraction. Apparently the technic of serum fractioning was not sufficiently developed at that time to enable Ascoli to concentrate the pseudoglobulin into a small volume as well as to separate it from the serum.

In his very extensive monograph on anthrax, Sobernheim (10) makes no mention of the fractionation of the immune serum, probably because the serum itself was satisfactory for most purposes (p. 696).

ANIMAL-INOCULATION TESTS

The protective power of the globulin concentrates was determined by inoculation experiments, mostly on guinea pigs, of which 263 were used. The preliminary experiments soon showed that the immune bodies were present in the globulin preparations. It is highly probable that the loss of immune bodies during the concentration was not very great; but an exact statement is not possible because at the present time there is neither a unit of anthrax toxin nor of immune body known. They have not yet been studied sufficiently to be standardized. The general statement may be made that the globulin concentrates are more potent than an equal volume of the corresponding serum. Only those details of the tests which are of special interest are mentioned. One of the main objects of the tests was to obtain data that might throw light on the problem of the nature of the immune bodies—whether they were different from or identical with the pseudoglobulin. The tests made so far are not easy to interpret, and little light is thrown by them on the problem.

In test 5, Table II, it was desired to ascertain whether a given weight of pseudoglobulin had the same protective power when present alone as in the globulin preparations, and when present with all the other serum constituents as in the serum administered.

In the first animal-inoculation tests the virus employed was a 24-hour bouillon culture of an attenuated strain of *Bacillus anthracis*, prepared by inoculating from an agar culture the amount of anthrax bacilli that can be taken up on a standard loop into a tube containing 10 c. c. of bouillon; 0.25 c. c. of such a culture constituted the dose. Later, however, a standardized suspension of anthrax spores in normal salt solution (4,000,000 spores per cubic centimeter) was employed, the dose also being 0.25 c. c. The injection of the serum and globulin preparations was made intraperitoneally, followed in 48 hours by a subcutaneous injection of the virus. Virus "Davis-C" represents a culture of *B. anthracis* uniformly fatal for guinea pigs and rabbits; "Davis–D" is fatal for guinea pigs but not rabbits; and "Chestertown" is fatal for sheep, cattle, and horses.

From the results obtained (Table II) on 3 lots of 12 guinea pigs, which had been injected with varying amounts of serum 48 and globulin 48, it would seem that a given weight of globulin had approximately

the same protective power whether present alone or in the serum. But the results obtained at the same time with serum 96 and globulin 96 do not substantiate this view. It is to be noticed that three guinea pigs that received 1 c. c. each of globulin 96, containing 0.147 gm. of pseudoglobulin, died before the close of the test, as well as three that received 1 c. c. each of the heated globulin 96, containing 0.150 gm. of pseudoglobulin. On the other hand, two guinea pigs out of three that had received 0.075 gm. of globulin (mostly pseudoglobulin, with a small amount of euglobulin) in the form of 1 c. c. of serum 96 survived. Likewise, two out of three survived after receiving 2 c. c. of serum 96. From these latter results alone the inference might be drawn that a protective action existed in the serum which was absent in the globulin preparations. The early death of practically all the guinea pigs that received globulin 96 (Table II) led to the suspicion that the virus C was too strong.[1] Accordingly test 5 was repeated, using the weaker virus D and increasing the quantities of globulin 96 injected (test 7, Table III); otherwise the two tests were the same. (For a description of the virus, technic of the injections, etc., see page 41.)

In test 7 (Table III) the results obtained with globulin 48 are practically the same as those obtained in test 5 (Table II). A larger number of guinea pigs did not survive, although a weaker virus was used. Plainly serum 48 protected a larger number of guinea pigs against virus D than against virus C. In test 7 the large number of survivals with serum 48 as compared with globulin 48 would indicate that the serum contained protective bodies which were absent in the globulin preparations. This is just the reverse of the result in test 5, in which the stronger virus was used. For serum 96 and globulin 96, the results in test 7 are essentially similar to those obtained in test 5, except that a few more of the globulin guinea pigs survived. It is apparent from the results that no definite statements can be made as to whether the survivals were due to the weaker virus or the increased amounts of globulin.

In the tests that were made the protective power of globulin 48 heated was as great as that of globulin 48 unheated, although the former was prepared from serum heated for six hours in a 60° C. air oven in the presence of one-third saturation ammonium sulphate. In so far as the flasks containing the mixtures to be heated were at room temperature when placed in the oven, it is practically certain that the temperature of 60° C. was actually reached inside the flasks only toward the end of the heating period. This method of heating was first used because it was desired to heat the mixture up to 60° C. without exposing any part of it to a temperature much higher than this. In general it was similar to the method of heating described by Banzhaf (2, p. 115). On the other hand, globulin 96, heated, was not as potent in protective power as the corre-

[1] When the tests were in progress the time of death of the animals was noted almost every hour, day and night. Little would be gained by inserting these figures into the tables.

sponding unheated preparation. Just why heat should be detrimental to one serum and apparently without effect on the other is difficult to say. It is possible that serum 48, being the more potent of the two, lost only a relatively small part of its total potency when heated; while serum 96, being comparatively weak, lost a relatively large part of its potency under the same treatment. At the present state of our knowledge there seems to be no need for the heat treatment, although future work may indicate its desirability.

TABLE II.—*Results of test 5 at 72 and 144 hours after the inoculation of guinea pigs with virus C. Seventy-two guinea pigs were inoculated with serum and globulin on Dec. 11, 1915, and with 0.25 c. c. of virus C on Dec. 13, 1915. Six additional guinea pigs (controls) received only virus* [a]

Serum 48.				Globulin 48.							
				Not heated.				Heated.			
Dose.	Globulin in dose.	Result.		Dose.	Globulin in dose.	Result.		Dose.	Globulin in dose.	Result.	
		72 hours.	144 hours.			72 hours.	144 hours.			72 hours.	144 hours.
C. c.	*Gm.*			*C. c.*	*Gm.*			*C. c.*	*Gm.*		
1	0.044	Died Lived. do. .	..... Lived. Do.	0.25	0.038	Died .. Lived do	..... Died. Do.	0.25	0.035	Died Lived . do	..... Died. Do.
2	.088	Died Lived ..do. . .do. . .	..... Died. Do. Do.	.50	.076	.do. . do . .do do. .	Died. Lived. Died. Lived.	.50	.070	Died Lived .do do	..... Lived. Do. Died.
3	.131	.do. . . . do. . ..do ..do. .	Do. Do. Do. Lived.	.75	.114	do .do. Died . Lived. .	Do. Do. ..... Died.	.75	.105	do . do do do	Do. Lived. Died. Do.
4	.175	..do. . ..do. .	Do.	1.00	.152	.do. . .	Lived.	1.00	.140	..do. . .	Lived.
Serum 96.				Globulin 96.							
C. c.	*Gm.*			*C. c.*	*Gm*			*C c.*	*Gm.*		
1	0.075	Lived.. do. . . .do. . . do	Died. Lived. Do. Died.	0 25	0.037	Died do. . . Lived..	.... Died.	0.25	0.037	Died .. . do . do .do	....
2	.151	.do. . do	Lived. Do.	.50	.074	Died.. ..do . Lived.	.... Died.	.50	.075	..do do. .	....
3	.226	Died. Lived ..do. . . do	..... Lived. Do. Do.	.75	.111	Died.. ..do. ..do. . . .do	....	.75	.112	..do . ..do . do .. do ..	....
4	.302	..do do. . .	Do. Do.	1.00	.147	do .. ..do ..	....	1.00	.150	do Lived..	..... Died.

[a] The six control guinea pigs died in less than 72 hours.
Globulins were prepared from blood drawn on November 5, 1915.
In serums 48 and 96 the globulin consisted largely of pseudoglobulin; the globulin preparations contained only pseudoglobulin (see page 39).
In making the heated preparations the serum was heated for six hours at 60° C.

If Tables II and III be divided horizontally by a line running between the 2 and 3 c. c. doses of serum, and the number of survivals in the upper half compared with those in the lower, it will be apparent that the protective action of neither serum nor globulin preparations was strictly proportional to the dose. In Table II there were 10 survivals in the upper

half and 13 in the lower. In Table III there were 18 survivals in the upper and 17 in the lower. These results indicate that the dose is only one of the many factors which decide the course of an individual test. They also indicate the desirability of large numbers of results before generalizations are made.

TABLE III.—*Results of test 7 at 72 and 144 hours after the inoculation of guinea pigs with virus D. Seventy-two guinea pigs were inoculated with serum and globulin on Jan. 15, 1916, and with 0.25 c. c. of virus D on January 17, 1916. Six additional guinea pigs (controls) received only virus* [a]

Serum 48.				Globulin 48.							
				Not heated.				Heated.			
Dose.	Globulin in dose.	Result		Dose.	Globulin in dose.	Result.		Dose.	Globulin in dose.	Result.	
		72 hours.	144 hours.			72 hours.	144 hours.			72 hours.	144 hours.
C. c.	*Gm.*	Lived.	Died.	*C. c.*	*Gm.*	Died	.....	*C. c.*	*Gm.*	Lived	Died.
1	0.044	..do...	Lived.	0.25	0.038	Lived.	Died.	0.25	0.035	..do..	Lived.
		..do..	Do.			..do...	Lived.			..do .	Do.
		..do...	Died.			Died..	.....			Died	.....
2	.088	..do...	Lived.	.50	.076	Lived	Died.	.50	.070	Lived.	Died.
		..do...	Do.			..do..	Lived.			..do. .	Do.
		..do...	Died.			.do..	Died.			Died	.....
3	.131	..do...	Lived.	.75	.114	..do...	Do.	.75	.105	Lived	Lived.
		..do...	Do.			do..	Lived.			Died	.....
		..do...	Do.			Died	.....			Lived	Died.
4	.175	..do...	Do.	1.00	.152	..do...	.....	1.00	.140	. do..	Lived.
		..do...	Do.			Lived.	Lived.			..do..	Do.

Serum 96.				Globulin 96.							
C. c.	*Gm.*	Died	.....	*C. c.*	*Gm.*	Lived	Died.	*C. c.*	*Gm.*	Died.	.....
1	0.075	Lived	Lived.	0.50	0.074	. do..	Lived.	0.50	0.075	. do.	.....
		..do..	Do.			..do..	Do.			Lived	Died.
		..do..	Do.			Died	.....			do..	Do.
2	.151	..do...	Do.	1.00	.147	..do	.....	1.00	.150	. do...	Lived.
		..do..	Do.			Lived	Lived.			.do..	Do.
		Died	.....			Died	.....			Died	.....
3	.226	Lived	Died.	1.50	.221	Lived	Lived.	1.50	.225	..do.	.....
		..do..	Lived.			..do..	Do.			Lived	Lived.
		..do .	Died.			..do..	Died.			Died	.....
4	.302	..do...	Lived.	2.00	.294	..do. .	Do.	2.00	.300	Lived.	Died.
		..do..	Do.			.do	Do.			..do...	Lived.

[a] Of the 6 control guinea pigs, 3 died in less than 72 hours and the remaining 3 died in less than 144 hours. Globulins prepared from blood drawn Nov. 5, 1915.

In serums 48 and 96 the globulin consisted largely of pseudoglobulin; the globulin preparations contained only pseudoglobulin (see p. 39).

In making the heated preparations the serum was heated for 6 hours at 60° C.

In test 6 the protective action of the globulin preparations on horses, calves, and sheep was demonstrated. The results are contained in Table IV.

All of the other globulin preparations were found to be potent when tested. For the reasons already stated, the protective power of the different preparations can be measured only approximately, and therefore the influence of heat, the relation between globulin contents and potency, etc., will remain in the problematic stage until standardized units of anthrax toxin and antitoxin are available for experimental purposes.

TABLE IV.—*Test 6: Results of inoculations of larger animals. The animals were inoculated with globulin on Dec. 17, 1915, and with virus "Chestertown" on Dec. 21, 1915*[a]

Animal No.	Dose of globulin.	Pseudo-globulin in dose.	Dose of virus.	Result.
		Gm.	*C. c.*	
Horse 132	5 c. c. globulin 48	0.760	0.5	Alive, Jan. 4, 1916.
Horse 130	5 c. c. globulin 48 (heated)	.700	.5	Do.
Horse 143	8 c. c. globulin 96	1.179	.5	Dead, Dec 30, 1915.
Horse 140	8 c. c. globulin 96 (heated)	1.202	.5	Dead, Dec. 31, 1915.
Horse 133	Control		.5	Dead, Dec. 27, 1915.
Calf 86	5 c. c. globulin 48	.760	.5	Alive, Jan. 4, 1916.
Calf 70	5 c. c. globulin 48 (heated)	.700	.5	Do.
Calf 87	8 c. c. globulin 96	1.179	.5	Do
Calf 69	8 c. c. globulin 96 (heated)	1.202	.5	Do
Calf —	Control		5	Do.
Sheep 507	5 c. c. globulin 48	.760	.25	Dead, Dec. 30, 1915.
Sheep 506	5 c. c. globulin 48 (heated)	.700	.25	Alive, Jan 4, 1916.
Sheep 505	8 c. c globulin 96	1.179	.25	Dead, Dec. 23, 1915.
Sheep 510	8 c. c. globulin 96 (heated).	1 202	.25	Do.
Sheep 502	Control		.25	Do

[a] In making the heated globulin preparations the serum was heated 6 hours at 60° C The globulins were prepared from blood drawn November 5, 1915.

During a recent outbreak of anthrax in a herd of animals near Richmond, Va., anthrax-globulin preparations were used with very good results. In the course of a week several cows had been lost on this farm and others were sick. An investigation showed the presence of anthrax infection. At this time three cows had high temperatures (104°–106° F.) and were manifesting severe symptoms of the disease. Twenty c. c. of globulin prepared from serum 48 were administered intravenously to each of the three animals. One of the animals was in a dying condition at the time the injection was made and died shortly afterwards. The next morning a decided drop in temperature was noted in the two other animals. Another injection of 20 c. c. of globulin was administered that afternoon. Complete recovery resulted in both cases. Prophylactic treatment was given to 244 head of cattle and 25 horses and mules, consisting of injections of 6 c. c. of globulin where that prepared from serum 96 was used, and 4 c. c. of the serum 48 preparation, administered simultaneously with 1 c. c. of a standardized anthrax-spore vaccine. Up to the present time no additional losses from anthrax have been reported in this herd.

CHEMICAL ANALYSES OF SERUM AND GLOBULIN PREPARATIONS

The first few analyses of serum and globulin were made by the methods described by Banzhaf and Gibson (3). These methods were found to be extremely laborious. If, after precipitating globulin by one-half saturation ammonium sulphate, the mixture was filtered through ordinary ashless paper, the filtrates were cloudy, and a sharp separation of precipitate from filtrate was uncertain. If filtered on hard paper (S. & S. 575), the filtrates were clear, but filtration was extremely slow and evaporation

probably great. It is believed that the use of the centrifuge, as described below, for the purpose of separating the globulin precipitate from the "filtrate" is a marked improvement over the method of filtering. The electrically driven centrifuge used had a revolving head of 12 inches diameter and could carry eight tubes of slightly more than 100 c. c. capacity. To effect a good separation, it must be run for 30 to 40 minutes at about 3,000 revolutions per minute.

In order that the results obtained may be comparable with those of Banzhaf, Gibson, and other investigators, the general method of analysis was similar to that used by them. The results contained in Table V and represented graphically in figure 1 were obtained by the following methods:

TOTAL COAGULABLE PROTEIN.—Ten c. c. of serum are pipetted into a 400 c. c. beaker, 300 c. c. of distilled water added and heated not quite to the boiling point. Two and one-half c. c. of $N/5$ acetic acid (1.2 per cent) are added. This flocculates the proteins at once. On account of the higher protein content of the globulin preparations, less than 10 c. c. may be taken, 5 c. c. being a convenient quantity. Less acetic acid should be used; 0.5 to 1 c. c. will flocculate the protein. After the flocculation has taken place, the solution is brought up to the boiling point for a minute, is allowed to cool, filtered on dry, weighed papers, then washed with small amounts of alcohol and ether, dried to constant weight at 100° C., and weighed. (Further details are given on page 48.) This is a simple, easy determination, and duplicates seldom differ more than 3 or 4 mgm. The filtrates should be water-clear or nearly so.

TOTAL GLOBULIN.—With a pipette 10 c. c. of serum (or globulin preparation or other product) is transferred to a centrifuge tube having a capacity of about 105 c. c. (size of tube 165 by 30 mm.). Forty c. c. of water and 50 c. c. of a saturated ammonium-sulphate solution are then added and centrifuged for 40 minutes at about 3,000 revolutions per minute. If the centrifuge runs smoothly, the precipitated globulin will be firmly packed to the bottom of the tube, leaving the supernatant fluid clear or faintly opalescent. Generally the supernatant fluid was not clear enough; it was then poured off into a second centrifuge tube and run again. On inverting the tube for complete drainage, none of the precipitate should be lost; it packs easily and firmly to the bottom. The volume of the supernatant fluid was noted, and the fluid was then rejected. This was done so that, if desired, corrections could be made for the fluid inclosed in the precipitate. The volume poured off was generally near 85 c. c., and varied between 80 and 90 c. c., according to the amount of precipitate. The corrections were not calculated, as their use at this stage would have been premature. The method can be still further improved before such corrections will be useful.

The globulin precipitates in the bottom of the centrifuge tubes are dissolved by the addition of distilled water, transferred to 400 c. c.

beakers, and heated as before to coagulate the protein. Only a small quantity of acetic acid is necessary for flocculation—usually 0.5 to 1 c. c. of the *N*/5 acid was sufficient. The precipitated protein is then filtered on weighed papers, washed till free, or almost free, from sulphate, then washed with small amounts of alcohol and ether, dried, and weighed as in the determination of total coagulable protein.

If desired, the globulin precipitate may be dissolved in 40 c. c. of water and again precipitated by the addition of 50 c. c. of saturated ammonium sulphate. This may free the precipitate of traces of albumin, but the loss of globulin at the same time probably makes this an unnecessary step.

Evaporation from the free surfaces of the fluids in the tubes during a 40 minutes' run in the centrifuge was found to be negligible, amounting to less than 1 or 2 c. c.

Good duplicates are easily obtainable. In 23 determinations the duplicates differed from 1 to 16 mgm., with an average of 7 mgm. This does not include a few determinations that were repeated because the duplicates differed enough to indicate error. Much depends upon the condition of the centrifuge. This must be a high-speed, smooth-running apparatus, which slows down smoothly. Practically the same results are obtained when the same serum is used for two globulin determinations about one month apart. The serums were preserved with 0.5 per cent chloroform (likewise the globulin preparations) and kept in a refrigerator.

The object of precipitating one volume of serum in a final dilution of 10 volumes of one-half saturated ammonium-sulphate solution is to prevent the contamination of the precipitate with albumin, which is said to be absorbed. This is the reason why the precipitate is dissolved and reprecipitated by some workers. While this procedure may be advisable for certain analytic purposes, it is objectionable when such results are to be used in connection with a study of antitoxin or similar products obtained by precipitating one volume of serum in a final dilution of three volumes of one-half ammonium-sulphate solution. This is one of the reasons why the analytic method has been further modified and improved so that the precipitation of globulin in the analyses and in the separation of large quantities of globulin for therapeutic use are both accomplished under the same conditions.

The filter papers used were S. & S. 589 "white ribbon," 15 cm. These were placed in weighing bottles, 50 by 40 cm., dried for six hours in an electrically heated air oven at 100° C., and weighed. They were then dried a second time for two hours and weighed to be certain that the weight was "constant." Almost invariably the second weighings differed from the first by 2 or 3 mgm. Drying the papers to an absolutely constant weight seldom occurred. Two empty weighing bottles were dried along with the others. Their weight, which was taken several times, varied only a fraction of a milligram. All weighings were to the nearest

milligram. The second weight of the paper and bottle was used, regardless of whether it was higher or lower than the first.

After being used for filtration, the papers containing a precipitate were replaced in their respective bottles, dried for 14 hours in the air oven at 100° C., and weighed. They were then dried a second time for two hours and weighed. It was found that 14 hours' drying almost always dried the papers and precipitates completely; the second weighing differed from the first only by 2 or 3 mgm. Occasionally, when the second weighing was less than the first by 6 mgm. or more, a third drying and weighing was made of the particular bottle. The lesser of the two weights was used. As is well known, the increase in weight after prolonged drying is due to oxidation.

A large number of blank filtrations, 23 in all, were made as follows: Through a weighed paper a filtrate obtained from a globulin or similar determination was passed. The paper was washed free from sulphate, then with alcohol and ether, dried 14 hours, and weighed as if it had contained a precipitate. The object was to ascertain the extent of the change in weight due to the mechanical handling, drying, etc. Out of the 23 blanks, the differences were 3 mgm., or less, in 18 blanks; generally there was a slight increase in weight. In the 5 other blanks the differences were 5, 5, 6, 6, and 7 mgm.; 3 of these were gains and 2 were losses in weight. These blanks were not used in correcting the weights of precipitates, as they were small enough to be negligible.

In so far as the weight of a precipitate is obtained by difference, it is obvious that care must be used to be certain that the conditions of drying, etc., are such as to lead to a minimum of error. It is probable that some investigators have not realized that errors of a few milligrams are almost unavoidable; one investigator using this method published results to the tenth of a milligram. It is believed that a minimum of error will result under the following conditions: If the first and second weights of the empty dry papers generally differ by 3 mgm. or less, either weight should be used consistently—that is, either the first weight should be used throughout or the second weight throughout, regardless of whether the differences are plus or minus. After drying the paper and precipitate, the minimal weight should be used, regardless of whether this is the first, second, or even third. Should differences of more than 3 mgm. appear often between consecutive weighings, it is probable that the drying was incomplete.

The analytic data obtained on the serums of horses 48 and 96 and mules 148 and 149 are contained in Table V, together with the data obtained on the globulin preparations. The data in Tables I to IV are obtained from those of Table V. It will be apparent from the last table that when a different technic is used in the determination of globulin in the same serum the results are different. Whenever several results were obtained on one serum that result obtained by single precipitation and centrifuging was regarded as correct. The variations from this

correct result are recorded because they indicate, to some extent, just how the results are affected by the differences in technic. For most purposes it is probable that any method will yield results that are comparable and useful. One of the objects of the repeated trials was to develop a technic by which it would be possible accurately to determine euglobulin, pseudoglobulin, and albumin separately in any one sample of serum, so that the sum of the three determinations would almost exactly equal the total coagulable protein in the same serum. Theoretically this should be true; practically the numerous sources of unavoidable error do not permit such accurate work.

TABLE V.—*Analytic data on serums and globulin preparations*

Date blood was drawn.	In 10 c. c. of—	Total coagulable protein.		Total globulin.[a]	Total globulin in total protein.
		Serum.	Globulin.[a]		
		Gm.	*Gm.*	*Gm.*	*Per cent.*
July 26, 1915	Serum 48	0.817		[b] 0.643	79
Do	Globulin 48		1.757		
Sept. 21, 1915	Serum 48	.767		[c] .503	66
Do	Globulin 48		1.420		
Do	Globulin 48 (heated)		1.365		
Nov. 5, 1915	Serum 48	.680		.438	64
		[d] .672		[d] .457	
Do	Globulin 48		1.520		
Do	Globulin 48 (heated)		1.400		
Jan. 6, 1916	Serum 48	.674		.411	61
Do	Globulin 48		2.110		
Do	Globulin 48 (heated)		2.110		
July 26, 1915	Serum 96	.815		[b] .649	80
Do	Globulin 96		1.704		
Sept. 21, 1915	Serum 96	.861		[c] .669	78
Do	Globulin 96		1.445		
Do	Globulin 96 (heated)		1.415		
Nov. 5, 1915	Serum 96	.887		.754	85
		[d] .893		[d] .744	
Do	Globulin 96		1.474		
Do	Globulin 96 (heated)		1.502		
Jan. 6, 1916	Serum 96	.789		.627	79
Do	Globulin 96		2.247		
Do	Globulin 96 (heated)		2.193		
Aug. 16, 1915	Serum 148	.752		[c] .419	56
Oct. 5, 1915	....do	.645		[c] .312	
				[e] .331	
				[f] .333	
				.352	55
Oct. 25, 1915	....do	.734		[b] .384	
				.438	60
Nov. 19, 1915	....do	.768		.455	59
Dec. 15, 1915	....do	.768		.455	59
		[d] .776		[d] .436	
Feb. 2, 1916	....do	1.047		.796	76
Mar. 6, 1916	....do	.907		.644	72
Aug. 16, 1915	Serum 149	.750		[e] .461	61
				[b] .369	
Oct. 5, 1915	....do	.673		[e] .340	
				[e] .337	
				[f] .366	
				.414	62
Oct. 25, 1915	....do	.793		[f] .492	
				.533	67
Nov. 19, 1915	....do	.745		.494	66
Dec. 15, 1915	....do	.730		.414	57

[a] All determinations of globulin are by single precipitation and centrifuging unless otherwise qualified.
[b] Globulin precipitated once and filtered.
[c] Globulin precipitated twice and filtered.
[d] Repetition of previous determination.
[e] Globulin by magnesium-sulphate saturation, twice precipitated and filtered.
[f] Globulin precipitated twice and centrifuged.

In general there was from three to four times as much globulin in the globulin preparations as there was in the same volume of serum. Thus, 10 c. c. of serum 48, January 6, 1916, contained 0.411 gm. of total globulin; about three-fourths of this was pseudoglobulin. The globulin concentrate prepared from this serum contained five times that amount of globulin, 2.110 gm., all of which was pseudoglobulin. This was determined by direct coagulation rather than by precipitation with ammonium sulphate, because the results are more accurate by the former method. Whether the globulin preparations were three or four times as potent in protective power as the same volume of serum can not be stated definitely, for reasons already given. The extent to which other proteins were removed during the globulin concentration is indicated in the last column of Table 1.

CHANGES IN THE SERUM PROTEINS DURING THE COURSE OF HYPERIMMUNIZATION

The changes in the serum proteins which take place during the course of hyperimmunization against diphtheria were studied by Hiss and Atkinson (6) and by Ledingham (7). Similar studies were made by Banzhaf and Gibson (3) on the plasma of horses immunized simultaneously against diphtheria and tetanus. They state (3, p. 203):

> The observations of Atkinson and Ledingham, so far as we are aware, are the only determinations of the quantitative relation of the serumglobulin content and antitoxic potency throughout the course of immunization. The subject is of extreme importance because of the constant association of the antisubstance with the serumglobulin.

Hartley's work on this subject (5) was published more recently (see p. 56).

The following quotation from the work of Banzhaf and Gibson (3, p. 206) will serve the purpose of briefly summarizing some of the more important results obtained by the above investigators:

> While the greatest rise in the serumglobulin was usually coincident with maximum antitoxic potency, as already pointed out, the extent of this increase in the serumglobulin was practically independent of the antitoxic potency when the results on more than one horse were contrasted. There may be, then, no relation between the absolute or percentage increase of the serumglobulin and the antitoxic potency in the plasma of different horses. The increase in the serumglobulin of refractory horses may surpass that in the plasma of some of those yielding a high antitoxin.

Obviously a study of anthrax serum during immunization was desirable for at least two reasons: (1) To ascertain whether the increase in potency was accompanied by an increase in globulin, and (2) in so far as the *Bacillus anthracis* does not form a soluble toxin while the bacilli of diphtheria and tetanus do, the serum changes would undoubtedly throw light on both the practical and theoretical knowledge of many related problems in immunity.

In figure 1 the analyses of the serums of horses 48 and 96 and mules 148 and 149 are plotted against the time of bleeding. The immuniza-

tion of the horses was begun in September, 1914, and continued with increasing doses of virus until June, 1915 (4, p. 9). Consequently at the time the analyses were made the serums were past the stage where the characteristic serum changes were to be expected. The immunization of the mules was begun in August, 1915, and mule 149 died on December 23, 1915. It is apparent from figure 1 that the changes in

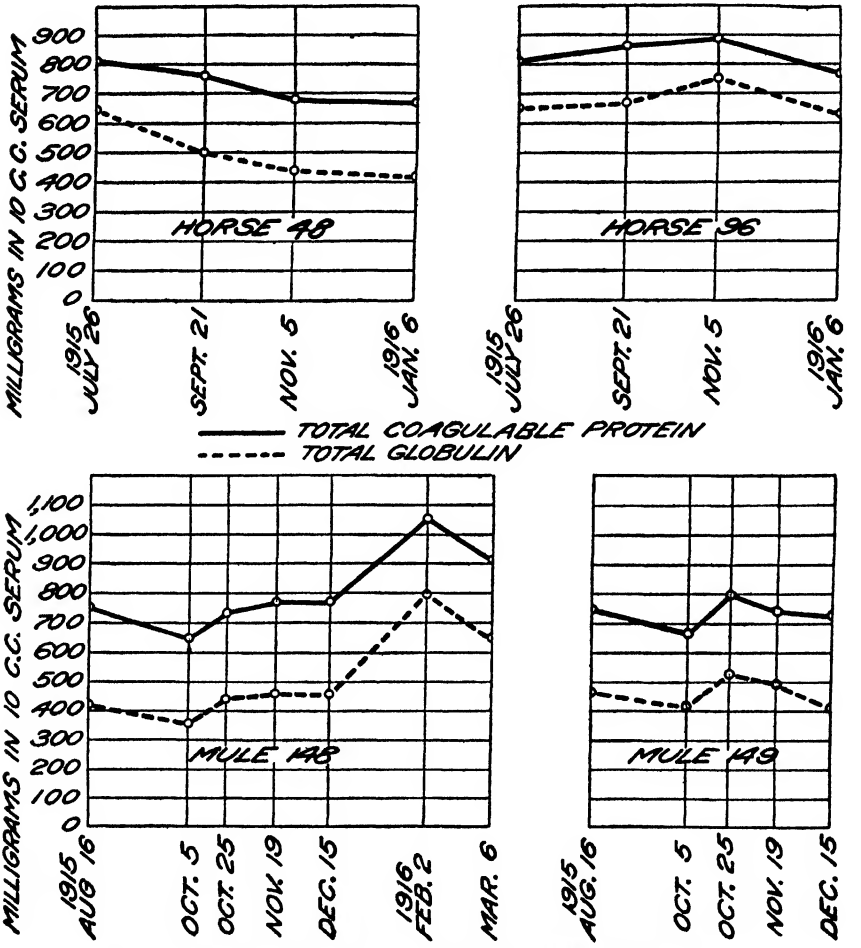

FIG. 1.—Changes in the serum proteins during the course of hyperimmunization.

the serum of mule 149 were similar to those in mule 148 shortly after the immunization was begun. The decline in the condition of mule 149 is believed to be the cause of the downward slope of the curve, or vice versa. The curve for mule 148 is typical in so far as it shows the drop in protein content in the beginning of the immunization, or the "negative phase," followed by a very pronounced rise in protein content. The total coagulable protein and the total globulin rose and fell together.

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The term "total globulin" is here used, as it is in Table V, to include the protein precipitated on 50 per cent saturation with ammonium sulphate. In so far as mule 148 received increasing doses of virus during the immunization, the statement may be made that with the rise in total protein and globulin there was a rise in potency; or at least the resistance of the mule was tremendously increased toward anthrax virus.

It would be unsafe to generalize from the curve for a single animal, but obviously in the case of mule 148 the presence of a soluble toxin was not necessary for the production of those serum protein changes usually noticed in diphtheria- and tetanus-immune serums.

Similar changes were observed by Hartley (5, p. 268) in immunization against rinderpest. The increase in total protein and total globulin during immunization has therefore been observed under the following five conditions:

(1) In diphtheria, in which a soluble toxin is involved.

(2) In tetanus, in which a soluble toxin is involved.

(3) In anthrax, in which no soluble toxin is involved according to the present state of our knowledge.

(4) In rinderpest, in which a filterable virus is involved.

(5) In diphtheria, when there is an increase of serum proteins without the production of antibodies.

TREATMENT OF ANTHRAX IN MAN

Since the beginning of the preparation of anthrax serum and globulin by the Bureau of Animal Industry, considerable amounts of these products have been furnished for the treatment of the disease in man, with highly satisfactory results.

Of the serum, the curative dose recommended is 40 c. c. administered subcutaneously in four or five places, repeated as necessary, after intervals of 24 hours, with injections of 25 c. c. In advanced cases the dose may be increased and the injections made intravenously. The dose of the globulin preparation is based on its proportional concentration and is usually from 10 to 15 c. c. The dose being considerably smaller than that of serum, the injection may be safely made intravenously. With other types of protein absent the globulin preparation is superior to the serum in that anaphylactic reaction is minimized.

A number of cases of anthrax in man were treated at Bellevue Hospital, New York, N. Y. Reports from this institution on several of these cases show that a marked lowering of the temperature and reduction of the edematous swelling followed the first injection of serum.

STANDARDIZATION OF ANTHRAX SERUM BY COMPLEMENT FIXATION

It has been found that the serum from various animals treated in an identical manner varies greatly in potency, so that one of the chief difficulties in the production of anthrax serum is its standardization, the various

methods so far employed being quite indefinite and the results variable. The writers have tried several methods of standardization. Sobernheim's method (10), consisting of the intravenous injection of graduated doses of the serum into a series of rabbits, followed immediately with subcutaneous injections of 0.001 loopful of a suspension of virulent anthrax bacilli in saline solution was the first method tried. This, however, did not prove as satisfactory as Ascoli's method. In this test a 24-hour-old attenuated bouillon culture is used, which is of such virulence that when introduced subcutaneously in a 0.25 c. c. dose will kill guinea pigs weighing 350 gm. each in from two to three days.

These cultures must be previously standardized in such a way that they will kill guinea pigs which 24 hours previously have been injected intraperitoneally with 2 c. c. of normal serum. Guinea pigs treated in the same manner and with the same dose of titrated standardized immune blood serum must remain alive. The testing of the serum is carried out on six guinea pigs, each receiving 2 c. c. of the serum intraperitoneally, followed in 24 hours with a subcutaneous injection of the established dose of the test culture. The serum is considered satisfactory for immunization purposes if at least four of the guinea pigs remain alive over six days while the control animals die in three or four days. This test has been modified by using graduated amounts of the serum under test.

The results obtained with the above methods are greatly influenced by the variance in individual susceptibility of the test animals, the character of the virus employed, etc. For this reason the writers have undertaken a series of experiments with the complement-fixation test, with a view to ascertaining its value in the standardization of anthrax serum, and while our work on this phase of the subject is only in the experimental stage and incomplete, the results so far are quite gratifying, and point to the possibility of employing this test as a means of more accurate standardization. So far the serum from two horses and a mule used by the Bureau of Animal Industry in the preparation of anthrax serum, as well as serum from horses in various stages of hyperimmunization obtained from various biological firms of the country, have been employed in this work. At the present time the serums of four horses under the course of hyperimmunization are being studied.

Several antigens have been tried, the best results being obtained with a bouillon culture of a slightly virulent strain of *Bacillus anthracis* (virulent for white mice and occasionally for small guinea pigs). Such a culture is grown at incubator temperature from three to five days and then placed in the refrigerator for two or three weeks or even longer. It is then heated at 60° C. for one-half hour and titrated against a known potent anthrax serum used as a standard.

In standardizing the serum the usual technic employed in the complement-fixation test applies, with the exception that varying quantities of the serum under test are used. So far in the work the writers have

been employing 12 tubes to a test, commencing with 0.2 c. c. of serum in the first tube and graduating the amount down to 0.005 c. c. in the last tube.

In the titration of the antigen and in all standardization tests the results are read at exactly one hour after the addition of the amboceptor and sheep cells, during which time the tubes were kept in the incubator at a temperature of 37.50° C.

Table VI illustrates some of the results obtained, the letters beneath the varying quantities of serum signifying, N, no fixation of complement; S, slight fixation; Gd, good fixation; and Pf, perfect fixation.

TABLE VI.—*Results of complement fixation tests with anthrax serum*

Sample.	Degree of fixation.											
Serum in tubes[a] ..... c. c.	0.2	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.005
A[b]	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.
B[c]	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.
C[d]	Pf.	Pf	Pf.	Pf.	Pf.	Pf.	Gd.	S.	N.	N.	N.	N.
D[e]	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Gd.	S.	N.	N.
E[f]	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.
F[g]	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Gd.	S.
G[h]	Pf.	Pf.	Pf.	Pf.	Gd.	Gd.	S.	N.	N.	N.	N.	N.
48[i]	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Pf.	Gd.	N.	N.
96[j]	Pf.	Pf.	Pf.	Pf.	Gd.	S.	N.	N.	N.	N.	N.	N.
Normal serum[k]	N	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.	N.

[a] Where the smaller quantities were used, the serum was first diluted to a known strength to avoid inaccuracies likely to occur where minute amounts are employed.
[b] Sample A. Serum from horse which had been vaccinated and subsequently infected with a minute amount of anthrax culture.
[c] Sample B. Same as A.
[d] Sample C. Serum from horse undergoing hyperimmunization. Had received growth from 18 agar slants five-eighths inch surface 17 days previous to the taking of this sample.
[e] Sample D. Serum from horse completely hyperimmunized.
[f] Sample E. Serum from horse which had merely been vaccinated against anthrax.
[g] Sample F. Serum from horse completely hyperimmunized.
[h] Sample G. Serum from hyperimmune horse which had been producing serum for over a year.
[i] Sample 48. Bureau of Animal Industry hyperimmune horse. Serum known to be of good potency through numerous field and laboratory tests.
[j] Sample 96. Bureau of Animal Industry hyperimmune horse. Serum known to be inferior to that from horse 48.
[k] Normal serum. Control.

The above serums were also employed in animal-inoculation tests, in which the results in general corresponded with the complement-fixation test.

While no definite conclusions can be drawn at this time, it appears from the results thus far obtained that as the process of hyperimmunization against anthrax progresses the complement-fixing action of the serum increases. Serum from different animals hyperimmunized by the Bureau of Animal Industry proved to be of different potency, one being of a high potency and the other of a comparatively low potency. (See Table VI.) On applying the complement-fixation test to these samples the complement-fixing value of the serum of highest potency was considerably higher than that of the other serum. Similar results have been obtained with serum from outside sources. A number of other samples of serum from horses in different stages of the hyperimmunization process were tested,

and so far the writers have found that the complement-fixing value has varied with the stage of hyperimmunization—that is, serum from animals in the early stages of the hyperimmunization process possesses little or no complement-fixing value, while that from animals in the advanced stages of the process exhibits considerable complement-fixing properties. The writers are hopeful that further work along this line will bear out the results thus far obtained.

Thus it may prove that by applying a known potent anthrax serum as a standard for the titration of the antigen and for establishing the minimum amount of serum which will cause complete fixation of complement in the presence of the determined amount of antigen, a standard may be established which will permit an accurate standardization of anthrax serum.

No fixation of complement occurs with the serum from animals vaccinated against anthrax or even after they have received the smaller infective doses of virulent culture, a positive reaction occurring only after the beginning of the administration of larger quantities of culture.

SUMMARY

(1) Anthrax serum was fractioned by the methods used in the preparation of diphtheria antitoxin. The anthrax antibodies were associated with the pseudoglobulin fraction.

(2) The globulin preparations contained the antibodies in a concentrated form. This was shown in numerous tests on laboratory animals. The preparations were likewise potent in tests on larger animals—that is, cattle, horses, etc. When administered to human beings (men) infected with anthrax, the globulin preparations were found to have great therapeutic value. However, no data have yet been obtained which permit accurate measurement of the potency of either the serum or the globulin obtained therefrom.

(3) The methods of analysis of serum and similar preparations of globulin have been improved by the use of the centrifuge instead of filtration as a means of separating globulin precipitates from their filtrates. The precipitates are obtained in compact form with a minimal amount of absorbed supernatant fluid. There is no need for reprecipitation.

(4) The changes in the amounts of the serum proteins in a mule undergoing immunization to anthrax were similar to those usually noted in the serum of animals being immunized to diphtheria, tetanus, and rinderpest—that is, there was a pronounced rise in the content of total coagulable protein and total globulin.

(5) Favorable results follow the use of anthrax serum or globulin preparations in the treatment of anthrax in man or animals. The globulin preparation is probably superior to the serum in the treatment of the disease in man, since the dose is smaller, and may be safely given intravenously, and the danger of anaphylaxis is minimized.

(6) The work on the standardization of anthrax serum by complement fixation, while still in an experimental stage and incomplete, points to the possibility of a more accurate means of standardization through its employment.

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CALCIUM COMPOUNDS IN SOILS

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INTRODUCTION

The term "chemical composition," as applied to soils, is frequently, perhaps it should be said generally, taken to mean the ultimate composition or total quantity of each of the elements present. If, however, a knowledge of the chemical composition of a soil is to be of any aid in studying chemical changes, the meaning of the term should be extended to include the kinds of compounds present—not only the quantity of each element present, but how they are combined.

The desirability of obtaining such information has been recognized by agricultural chemists for a long time, as is evidenced by the numerous methods that have been proposed by which soil compounds containing certain elements could be classified. Methods involving digestion with dilute acids, methods for the determination of humus, for organic phosphorus and organic sulphur, as well as methods for determining "availability," all belong to this category. None of these methods have been aimed at the identification of any definite compound, but rather at a grouping of compounds having some property in common. This is, however, a step in the direction of identification.

Without implying any limitation as to methods applicable to the determination of kinds of compounds in soils, two methods may be mentioned here: The petrographic and the analytical.

The use of the petrographic microscope in soil investigations has been fully discussed in a previous publication from this Bureau,[1] and it is only necessary to state here that this method has a serious limitation in that as at present developed it is not applicable to particles smaller than silt (0.005 mm. in diameter), and throws no light on the chemical nature of the fine material in the soil that no doubt is most active in any chemical changes that may take place.

The petrographic method is, however, a valuable adjunct to any other method that may be applied to the determination of the chemical nature

[1] McCaughey, W. J., and Fry, W. H. Microscopic determination of soil-forming minerals. U. S. Dept. Agr. Bur. Soils Bul. 91, 100 p., 11 fig. 1913.

of the compounds in soils, and may be used both to suggest other methods of attack and to confirm results obtained by other means.

The application of analytical methods to the problem of determining the identity and quantity of soil compounds presupposes some knowledge of these compounds. The method has the limitation common to all analytical operations—namely, that the differences sought to be determined may be smaller than the unavoidable error in the method used, and, furthermore, that the value of the results obtained depends on the accuracy of the information regarding the kind of compounds present.

It has seemed that information regarding compounds of calcium in the soil might be more satisfactorily obtained by the analytical method than in the case of other elements, because (1) there is available considerable information regarding the kinds of calcium compounds that may be present in soils, and (2) among these compounds several classes are represented having quite different properties and capable of being separated by analytical means.

The calcium compounds known or supposed to be present in soils are as follows: Calcium carbonate, or calcite; calcium magnesium carbonate, or dolomite; calcium sulphate, or gypsum; calcium phosphate with calcium chlorid or fluorid, apatite; and calcium silicates, a class represented by a number of minerals, nearly all containing metals in addition to calcium. All of these are known to occur in the rocks from which soils are formed, and all have been shown to be present in soils. In addition to these, most soils are supposed to contain compounds of calcium with the organic bodies commonly designated as humus. The assumption of the presence of such calcium-humus compounds is based, first, on the fact that when such humus bodies are isolated from soils they apparently combine with lime, forming compounds insoluble in water or dilute alkalies; and second, in the case of many soils such humus bodies can be leached from soils by dilute alkalies only after leaching with dilute acid, whereby the calcium is removed as a salt of the acid used, the humus bodies remaining in a condition to be leached by dilute alkali. With this information the calcium compounds present or likely to be present in soils may be classed as follows: Calcium as carbonate, as sulphate, as phosphate, as silicates, and as combined with humus compounds.

The present paper presents results obtained in attempting to classify the calcium compounds in soils as just outlined by the application of analytical methods supplemented by such information as was furnished by the petrographic method.

SOILS

The samples of soil selected for this work, 63 in number, represent 23 soil types from 24 locations in 19 States. Each location is represented by from two to four samples, according to the number of layers encoun-

tered: soil and subsoil; soil, subsoil, and deep subsoil; or soil, subsurface subsoil, and deep subsoil.

The character of the samples, location, and type name are given in Table I, in which the soils are classified according to the color of soil and subsoil.

TABLE I.—*Classification and characteristics of soils and subsoils according to their color*

CLASS I.—BROWN SOILS WITH LIGHT-BROWN TO YELLOWISH BROWN SUBSOILS

No.	Depth.	Type.	Location.
	Inches.		
1 (soil)	0–18	Holland sandy loam	San Diego County, Cal.
2 (subsoil)	18–40	do	
3 (soil)	0–10	Gloucester fine sandy loam	New London County, Conn.
4 (subsoil)	10–36	do	
5 (soil)	0– 9	Sassafras loam	Freehold Area, N. J.
6 (subsoil)	9–36	do	
7 (soil)	0– 8	Collington loam	Do.
8 (subsoil)	8–21	do	
9 (lower subsoil)	21–36	do	

CLASS II.—LIGHT-BROWN SOILS WITH YELLOWISH BROWN, MOTTLED, COMPACTED SUBSOILS

10 (soil)	0– 8	Spencer silt loam	No. Central Recon., Wis.
11 (subsurface)	8–24	do	
12 (subsoil)	24–36	do	
13 (lower subsoil)	36–72		
14 (soil)	0–12	Helmer silt loam	Latah County, Idaho.
15 (subsoil)	12–30	do	
16 (lower subsoil)	30–36		
17 (soil)	0– 6	Helmer loam	Montana forested region.
18 (subsoil)	6–20	do	

CLASS III.—RED OR REDDISH SOILS WITH RED SUBSOILS

19 (soil)	0– 5	Susquehanna clay	Montgomery County, Ala.
20 (subsoil)	5–36	do	
21 (soil)	0–12	Cecil clay loam	Troupe County, Ga.
22 (subsoil)	12–36	do	
23 (soil)	0– 6	Colts-neck loam	Freehold Area, N. J.
24 (subsoil)	6–36	do	
25 (soil)	0– 4	Mecklenburg clay loam	Mecklenburg County, N. C.
26 (subsoil)	4–24	do	
27 (lower subsoil)	24–36	do	

CLASS IV.—DARK-BROWN SOILS WITH BROWN TO YELLOWISH BROWN SUBSOILS AND A HEAVY CLAY HORIZON IN SUBSOIL

28 (soil)	0– 7	Grundy silt loam	Grundy County, Mo.
29 (subsoil)	7–18	do	
30 (lower subsoil)	18–36	do	
31 (soil)	0– 8	do.	Polk County, Nebr.
32 (subsoil)	8–36	do	
33 (soil)	0– 7	Charitan silt loam	Grundy County, Mo.
34 (subsoil)	7–18	do	
35 (lower subsoil)	18–36	do	

CLASS V.—DARK-BROWN TO BLACK SOILS WITH YELLOW-BROWN TO GRAYISH SUBSOILS

36 (soil)	0–20	Marshall silt loam	Pottawattamie County, Iowa.
37 (subsoil)	20–36	do	
38 (soil)	0–17	Barnes silty clay loam	Barnes County, N. Dak.
39 (subsoil)	17–48	do	

TABLE I.—*Classification and characteristics of soils and subsoils according to their color*—Continued

CLASS VI.—DARK-GRAY TO BLACK SOILS, WITH GRAY MOTTLED POORLY DRAINED SUBSOIL

No.	Depth.	Type.	Location.
	Inches.		
40 (soil)	0–6	Clyde silty clay	Wells County, Ind.
41 (subsoil)	6–16	do	
42 (lower subsoil)	16–36	do	
43 (soil)	0–6	do	Do.
44 (subsoil)	6–16	do	
45 (lower subsoil)	16–36	do	

CLASS VII.—LIGHT-BROWN TO GRAY SOIL, GRAY SUBSURFACE, AND HEAVY, PLASTIC, MOTTLED SUBSOIL

46 (soil)	0–8	Crosby silty clay loam	Grant County, Ind.
47 (subsoil)	8–12	do	
48 (lower subsoil)	12–36	do	
49 (soil)	0–6	Cherokee silt loam	Cherokee County, Kans.
50 (subsurface)	6–16	do	
51 (subsoil)	16–28	do	
52 (lower subsoil)	28–40	do	

CLASS VIII.—RED SOILS WITH RED SUBSOILS, HEAVY AND PLASTIC IN FLAT AREAS

53 (soil)	0–15	Vernon very fine sandy loam	Oklahoma Experiment Station.
54 (subsoil)	15–36	do	

CLASS IX.—LIGHT-BROWN TO GRAY SOILS WITH HEAVY CLAY SUBSOIL

55 (soil)	0–4	Oktibbeha clay	Oktibbeha County, Miss.
56 (subsoil)	4–36	do	

CLASS X.—LIGHT BROWN SOILS, LIGHT-BROWN TO GRAY SUBSOIL

57 (soil)	0–12	Ritzville silt loam	Franklin County, Wash.
58 (subsoil)	12–36	do	
59 (lower subsoil)	36–72	do	

CLASS XI —BROWN SOILS WITH HEAVY, PLASTIC YELLOW SUBSOIL

60 (soil)	0–4	Iredell clay loam	Chester County, S. C.
61 (subsoil)	4–30	do	

CLASS XII —LIGHT-BROWN SOIL, FAINT REDDISH BROWN UPPER SUBSOIL, AND MOTTLED LOWER SUBSOIL

62 (soil)	0–9	Pheba fine sandy loam	Starkville, Miss.
63 (subsoil)	9–36	do	

A short description[1] of these soil types follows.

CLASS I

HOLLAND SANDY LOAM.—The Holland sandy loam is a brown sandy loam, medium to rather coarse in texture. Organic matter is usually deficient. The subsoil is a brown to light-brown, open, friable sandy loam underlain by the parent rock, which is usually granite. The

[1] Prepared by Mr. T. D. Rice, Inspector of Soil Survey, Bureau of Soils.

topography is rolling and the drainage is good, sometimes even excessive. The type is usually timbered, and very little of it is under cultivation. The better portions are adapted to fruit and general farming crops.

GLOUCESTER FINE SANDY LOAM.—The soil of this type is a brown, fine sandy loam 6 to 12 inches deep. The subsoil is a fine sandy loam, yellowish brown as far as aeration and oxidation has extended. Beneath that lies the bluish glacial till derived from crystalline rocks. In the fine sandy loam oxidation has extended to a depth of 2 feet or more. The lower portion of the subsoil is usually coarse in texture. The type is derived from granitic drift, and glacial bowlders and gravel are found throughout the soil section. The topography varies from undulating to rolling, and the drainage is good. This type is regarded as a good farming soil.

SASSAFRAS LOAM.—This type consists of a light-brown loam underlain by reddish yellow to yellow heavy loam to silty clay loam of a moderately friable structure. At 28 inches coarser material consisting of a yellowish red to reddish-yellow sandy loam is encountered. This type occurs upon terraces in the Coastal Plain, and the drainage is always good. It is one of the best farming soils in that region and is adapted to general farm crops.

COLLINGTON LOAM.—This soil is a brown loam or heavy loam having a faint reddish to greenish cast. Below 10 to 14 inches the subsoil is a greenish brown, moderately friable clay loam or clay. This soil is derived from green sand deposits of the Coastal Plain. It is a productive soil well suited to general farm crops.

CLASS II

SPENCER SILT LOAM.—The surface soil to an average depth of 10 inches consists of a grayish brown silt loam. The subsoil is a yellowish brown to gray silt loam which becomes heavier with depth and grades into a silty clay loam at 16 to 20 inches. Below this the color of the lower subsoil is a yellowish brown to yellow mottled with gray, and the texture is usually heavier and more compact than the upper subsoil. The topography varies from undulating to gently rolling. The type was derived by weathering from a silty layer overlying the granitic drift. It is regarded as a good general-farming and dairying soil.

HELMER LOAM AND SILT LOAM.—This soil is a pale-yellow or light-brown loam or silt loam of rather compact structure 6 to 12 inches deep. The upper subsoil is of a lighter yellow color, but is similar in texture and structure to the soil. The lower subsoil below 24 to 30 inches is a pale-yellow or grayish yellow material of similar or heavier texture, forming a compact hardpan. The type is derived from wind-laid material. The topography is undulating to rolling, and drainage is well established. But little of the type is cleared and cultivated. Small grains and timothy are grown to a limited extent.

CLASS III

SUSQUEHANNA CLAY.—This soil to a depth of 3 to 6 inches is a stiff, red, sandy clay. The subsoil to a depth of 3 feet or more is a bright-red or yellowish heavy clay mottled with lighter colors. The subsoil is very impervious and the type as a whole does not retain moisture. The type is usually regarded as having a rather low agricultural value.

CECIL CLAY LOAM.—The surface soil is a bright-red, reddish gray, or reddish brown sandy clay loam 5 to 12 inches deep. The subsoil is a heavy, red, friable clay to a depth of 3 feet or more. Mica flakes and sharp fragments of quartz are found throughout the subsoil. This type is residual, being derived by weathering from gneiss and granite. The topography is rolling and the drainage is good. This is a valuable soil for general-farming purposes.

COLTS-NECK LOAM.—The soil of this type is a brownish red to reddish brown loam 8 to 10 inches deep. The subsoil is a dull-red clay loam which grades into a moderately friable and somewhat sandy clay. The type occupies round knolls. Ferruginous rock of reddish color is common in the substratum. Colts-neck loam is Collington loam in an advanced stage of oxidation.

MECKLENBURG CLAY LOAM.—The soil to a depth of 8 inches is a reddish brown to red clay loam. The subsoil is a yellowish brown or yellow, stiff, plastic clay. At 18 to 24 inches the partially decomposed parent rock is encountered. Iron concretions occur on the surface. The topography varies from undulating to rolling. Surface drainage is usually good. This type is residual soil derived from the diorite and gabbro principally. This is one of the best soils in the Piedmont, and good crops of cotton, wheat, and corn are secured. Of late years it has become noted as an alfalfa soil.

CLASS IV

GRUNDY SILT LOAM.—The soil of this type is a slate loam ranging in color from dark brown to black, but this is underlain at about 8 inches by light-gray or ashy-gray silt loam. This light-gray layer gradually becomes heavier in texture downward. At 18 inches it is underlain by a dark-brown or dark-drab, heavy, tenacious clay, mottled with yellowish brown spots. At 24 inches it becomes more silty and assumes a drab or light-gray appearance. The type occupies almost level to gently rolling areas. It is derived by weathering from silty or silty clay layers overlying a coarser drift sheet. The soil is very productive, and large yields of corn, wheat, and oats are secured.

CHARITON SILT LOAM.—This soil is a dark-brown silt loam which becomes heavy in texture with depth. At 8 inches mottlings of lighter gray begin which increase until the soil is a light ashy-gray color. This is underlain at depths of 8 to 15 inches by a dark-gray or dark brownish gray clay mottled with yellow. Below 26 inches the mottling becomes

less pronounced and, the texture is more silty. The type occupies terraces above the present flood plain. The soil material is an old alluvial deposit derived from drift and modified by weathering on a nearly level surface. The type is regarded as a good soil for general farm crops. The Chariton silt loam is the same as Grundy silt loam, but lies on river terraces.

CLASS V

MARSHALL SILT LOAM.—The surface soil is a dark-brown to black silt loam 20 inches deep. The subsoil is a yellowish brown silt loam or heavy silt loam usually more compact than the surface soil. Both soil and subsoil are calcareous, and lime concretions are abundant. The topography is almost level to rolling or hilly. The type is derived by weathering from loess. It is an excellent general-farming soil and is especially adapted to corn.

BARNES SILTY CLAY LOAM.—This type consists of a dark-brown to black silty clay loam underlain by gray to yellowish gray silty clay loam. The lower subsoil usually becomes more clayey. The topography varies from undulating to rolling. Natural drainage is fairly good. This type is derived by weathering from calcareous drift. It is a highly productive soil, and the greater part is sown to small grains.

CLASS VI

CLYDE SILTY CLAY.—This type consists of a dark-brown to black silty clay 6 to 12 inches deep underlain by a drab or gray and drab mottled clay subsoil. The topography is level, and the natural drainage is poor. This type is derived from moderately calcareous drift weathered under poor drainage conditions. When reclaimed, it is a good soil for general farming and dairying.

CLASS VII

CROSBY SILTY CLAY LOAM.—This type to a depth of 5 to 8 inches is a heavy silt loam varying in color from light gray to brownish gray. The upper subsoil to a depth of 10 to 12 inches is an ashen-gray to light-gray silty loam mottled with spots of yellowish brown clay. The lower subsoil is a yellow and gray mottled silty clay passing into a brown, tough, compact silty clay. Small iron concretions occur on the surface and through the soil and upper subsoil. The topography ranges from flat to slightly undulating. Natural drainage is imperfect. The type is derived by weathering from rather calcareous drift. Oats and hay are the chief crops grown on this type.

CHEROKEE SILT LOAM.—The soil of this type to a depth of 6 to 12 inches consists of an ashen-gray floury silt loam. The subsoil is a silt loam more compact than the surface material and nearly white in color. This extends to a depth of 14 to 18 inches, below which is found a tough, waxy, plastic heavy clay of a dark-drab color usually mottled with red. The topography is flat to undulating. The natural drainage is defi-

cient. The type is derived by weathering from argillaceous shales. Crop yields are poor, and much of the type is uncultivated.

CLASS VIII

VERNON VERY FINE SANDY LOAM.—This type consists of a very fine sandy loam 15 inches deep, overlying the subsoil of lighter red color and heavy texture. The topography is rolling to hilly and the drainage is good or even excessive. The type is of residual origin, being derived mainly from the Permian red sandstone. Where the soil has not been eroded, corn, wheat, and kafir give good yields.

CLASS IX

OKTIBBEHA CLAY.—This type consists of a heavy yellowish brown sandy clay to a depth of 3 feet or more. The topography is rolling to hilly, and the heavy soil does not absorb the rainfall; consequently erosion is excessive. Very little of the type is now under cultivation. Cotton is the principal crop, but yields are low.

CLASS X

RITZVILLE SILT LOAM.—The surface soil is a light-brown silt loam of smooth texture and rather compact structure. The subsoil to a depth of 6 feet or more is of similar texture and of slightly lighter brown color. The topography is undulating to rolling, and drainage is well developed. The parent material consists of loessial deposits having a rather calcareous composition. The type is devoted mainly to the production of wheat under dry farming.

CLASS XI

IREDELL CLAY LOAM.—The soil to an average depth of 4 inches is a dark-brown fine loam to clay loam. Quartz fragments are scattered over the surface, and small iron concretions are found throughout the soil. The subsoil is a heavy, sticky, plastic clay usually of a dark yellowish brown color. At a depth of usually 2½ to 3 feet soft, partly weathered rock is encountered. The surface varies from level to gently rolling. On account of the impervious nature of the subsoil, drainage is poor. The type is derived by weathering from diorite and other dark basic rocks. Where properly drained and cultivated, this soil is very productive, being adapted to cotton, corn, wheat, and alfalfa.

CLASS XII

PHEBA FINE SANDY LOAM.—This soil is a gray to light-brown fine sandy loam 6 to 10 inches deep. The subsoil is a yellowish brown, heavy, fine sandy loam which gradually passes into fine sandy clay or silty clay. The lower subsoil is usually mottled with reddish yellow and gray colors. A heavy clay substratum is encountered at from 3 to 6 feet. The surface varies from flat to gently rolling. Drainage is required over

the greater part of the type. Good crops of cotton, corn, peanuts, and cowpeas are secured under proper treatment.

PETROGRAPHIC EXAMINATION

Preliminary to chemical work, the samples were examined under the petrographic microscope for the presence or absence of the most important calcium-bearing minerals. The minerals sought for and their formulas are as follows:

Calcite.....$CaCO_3$.
Dolomite.....(Ca, Mg) CO_3.
Hornblende...Chiefly Ca (Mg, Fe)$_3$ Si_4O_{12} with $Na_2Al_2Si_4O_{12}$ and (Mg, Fe)$_2$ (Al, Fe)$_4$ Si_2O_{12}.
Augite.....Chiefly $CaMgSi_2O_6$ with (Mg, Fe) (Al, Fe)$_2$ SiO_6 and occasionally alkalies.
Gypsum.....$CaSO_4+2H_2O$.
Apatite.....(CaF)$Ca_4(PO_4)_3$ or (CaCl)$Ca_4(PO_4)_3$.
Plagioclase....Isomorphous mixtures of $NaAlSi_3O_8$ and $CaAlSi_2O_8$.
Epidote.....$HCa_2(Al, Fe)_3$ Si_3O_{13}.
Titanite.....Ca Ti SiO_5.

	II	III	II	III
Garnet.....	R_3.	$R_2(SiO_4)_3$.	R=Ca, Mg, Fe, Mn.	R=Al, Fe, Cr, and rarely Ti.

Zoisite.....$Ca_2(AlOH)$ Al_2 $(SiO_4)_3$.

The results are presented in Table II, in which P indicates that the mineral designated is present plentifully (approximately above 5 per cent); S that it is present in small quantities (scarcely above 3 per cent); VS that it is present in very small quantities (1 per cent or less); and T that it is present in traces. In a few instances the examination indicated that a mineral was probably present, but its identification was not satisfactory. This is indicated by T?.

TABLE II.—*Petrographic analysis of soils for calcium-containing minerals*

Class and No.	Calcite.	Dolomite.	Hornblende	Augite.	Gypsum.	Apatite.	Plagioclase.	Epidote.	Titanite.	Garnet.	Zoisite.
Class I:											
1			S		T		S				
2			S				S	S			
3			S				S	T	T	T	
4			S				T	T	T	T	
5			S				T	T			
6	T		S				T	S			T
7			S				S	S			
8	T?		VS					VS			
9			T				VS	T			
Class II:											
10			S				S	S			
11			S				S	S			
12			S				T	T	T		
13	T?										
14			S							S	
15			S				S	S	S	S	
16			S				S	S	S		
17			T				S	S			
18			VS				VS	VS			

TABLE II.—*Petrographic analysis of soils for calcium-containing minerals*—Continued

Class and No.	Calcite.	Dolomite.	Hornblende.	Augite.	Gypsum.	Apatite.	Plagioclase.	Epidote.	Titanite.	Garnet.	Zoisite.
Class III:											
19	T										
20			T								
21											
22											
23											
24											
25			T					S			
26								S			
27							T	S			
Class IV:											
28			T								
29			T				T			T	
30			S					S			
31			S				S	S		S	
32			S				S	S	T		
33			T								
34											
35			T				S	S			
Class V:											
36			S					S			
37		T	S				T	S	T		
38			S				S	T		T	
39	P		S		T?		S				
Class VI:											
40			S				S				
41			S				T				
42			S				S				
43			S				S				
44			S				S	S			
45			S				T				
Class VII:											
46			S				S	S			
47			S				S	S			
48	P		S				S			S	
49			S				T	T			
50							T	T			
51			T?								
52											
Class VIII:											
53							S	S			
54							S	S			
Class IX:											
55			VS								
56	T		VS								
Class X:											
57			VS	T			T			T	
58	T	T	VS	T			T				
59	T	T	VS	T			T				
Class XI:											
60			S				S	S			
61			S	S			S	S			
Class XII:											
62						T					
63	T		VS								

EXAMINATION BY ANALYTICAL METHOD

In attempting to distinguish between calcium compounds in these soils by analytical data, the following determinations were made: Total calcium; acid-soluble calcium by two methods, using different strengths of acid; carbonic acid; water-soluble calcium; and water-soluble sulphates.

METHODS OF ANALYSIS

The sample, after the removal of gravel and organic débris by sieving if necessary, was ground in an agate mortar, passed through a 100-mesh sieve, and dried at 105° C.

The total calcium was determined by fusion with sodium carbonate, acidifying, removing the silica in the usual way and precipitating the calcium as the oxalate after the removal of iron and alumina, the usual precaution of double precipitation being taken.

The acid-soluble calcium was determined by two methods designated "A" and "B," respectively.

In method A 5 gm. of soil were digested with 50 c. c. of 4 per cent hydrochloric acid for 12 hours, filtered, washed free of acid, and the calcium determined in the solution in the usual way by precipitation as oxalate.

In method B 10 gm. of soil placed on a dry filter paper in a funnel were leached with 2 per cent hydrochloric acid and washed free of acid. Where no effervescence was observed, two portions of 20 c. c. each of acid were used; where effervescence was observed, successive portions of 20 c. c. of acid were used until the evolution of carbon dioxid was no longer observed, and the soil was then washed free of acid. The calcium was determined as in method A.

Carbon dioxid was determined in a Knorr apparatus, being absorbed in a Geissler potash bulb and determined by the increase in weight. Water-soluble calcium oxid and sulphur trioxid were determined by precipitation from a water extract obtained by shaking 25 gm. of soil with 250 c. c. of water for 2 hours, allowing it to stand for 12 hours, and filtering.

The analytical data obtained in this way are presented in Table III. In conformity with the usual practice of recording soil analyses, calcium is stated in all cases as CaO, sulphates as SO_3 and carbonic acid as CO_2 rather than as the ions Ca, SO_4, and CO_3.

TABLE III.—*Partial analytical examination of soils in Classes I to XII for calcium*

Class and No.	Total calcium oxid.	Water-soluble calcium oxid.	Acid-soluble calcium oxid.		Carbon dioxid.	Water-soluble sulphur trioxid.
			Method A.	Method B.		
Class I:	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
1	3.18	.....	0.17	0.11	0.04	.....
2	2.98	Trace...	.21	.12	.09	Trace.
3	2.70	.05	.31	.21	.20	.02
4	2.77	.03	.12	.04	.10	.....
5	.70	.02	.28	.24	.25	.02
6	.48	Trace...	.14	.12	.19	.02
7	1.00	...do....	.34	.29	.12	Trace.
8	.55	.02	.17	.10	.13	.03
9	.63	.02	.10	.06	.15	.02
Class II:						
10	1.26	.01	.25	.21	.16	.01
11	1.11	Trace...	.12	.08	.11	.01
12	1.29	...do....	.14	.11	.04	Trace.
13	.96	...do....	.73	.41	.07	Do.
14	1.41	.05	.14	.10	.19	.03
15	1.34	.02	.19	.14	.16	.02
16	1.26	.03	.16	.12	.11	Trace.
17	.57	Trace...	.08	.05	.09	Do.
18	1.12	.02	.04	.02	.26	Do.
Class III:						
19	.98	.04	.79	.56	.07	Trace.
20	.96	Trace...	.86	.47	.12	.....
21	.49	...do....	.16	.10	.24	.03
22	.59	.. do....	.07	.04	.19	.....
23	.59	.02	.26	.23	.17	.02
24	.46	.02	.16	.13	.30	.02
25	1.98	Trace...	.14	.12	.11	.02
26	1.72	..do....	.21	.16	.19	Trace.
27	3.88	...do....	.68	.45	.12	.02
Class IV:						
28	.77	.02	.46	.37	.13	Trace.
29	.53	Trace...	.26	.21	.05	.01
30	1.10	.....	.54	.36	.12	Trace.
31	1.37	Trace.	.43	.39	.14	Do.
32	1.34	...do	.49	.39	.07	Do.
33	.82	...do....	.26	.24	.10	.01
34	.83	...do....	.22	.17	.19	.01
35	.87	.....	.47	.27	.07	.04
Class V:						
36	1.46	.02	.49	.38	.13	.02
37	1.92	.02	.94	.60	.38	.01
38	1.70	.03	.61	.52	.21	.01
39	6.58	.03	5.72	5.69	5.53	.02
Class VI:						
40	1.53	.02	.95	.81	.18	.01
41	1.48	.04	.77	.38	.17	.02
42	1.26	.03	.67	.44	.12	.01
43	1.40	.05	.89	.43	.15	.02
44	1.29	.02	.78	.58	.10	.02
45	1.14	.....	.81	.44	.06	.02
Class VII:						
46	.98	Trace...	.37	.28	.14	.03
47	.80	.04	.26	.20	.16	.02
48	6.15	.09	5.57	5.52	5.79	.02
49	.60	Trace...	.11	.09	.06	.02
50	.48	.....	.08	.07	.07	.02
51	.55	.02	.29	.18	.06	.13
52	.72	.02	.23	.06	.08	.18

TABLE III.—*Partial analytical examination of soils in Classes I to XII for calcium*—Continued

Class and No.	Total calcium oxid.	Water-soluble calcium oxid.	Acid-soluble calcium oxid.		Carbon dioxid.	Water-soluble sulphur trioxid.
			Method A.	Method B.		
Class VIII:	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
53	0.61	0.01	0.20	0.17	0.12	0.02
54	.59	Trace...	.19	.16	.07	.01
Class IX:						
55	.65	.05	.45	.40	.36	.01
56	.43	Trace...	.10	.06	.09	.....
Class X:						
57	3.05	Trace...	.47	.36	.05	Trace.
58	5 57	.03	3.04	2.50	1.97	.01
59	5.44	.02	3.10	2.72	2.14	.02
Class XI:						
60	4.43	.01	.17	.10	.09	.01
61	4.64	Trace...	.30	.19	.05	.....
Class XII:						
62	.27	Trace...	.02	Trace...	.08	.....
63	.37	.....	.03	.01	.05	.....

INTERPRETATION OF THE ANALYTICAL DATA

In interpreting the analytical data in terms of calcium compounds the following assumptions have been made and procedure followed:

It has been assumed that leaching with 2 per cent hydrochloric acid (method B) decomposed all calcium carbonate and all combinations of calcium with humus bodies, and that such leaching and subsequent washing with water dissolved all calcium sulphate. The resulting solution then obtained by method B would contain all the calcium present as carbonates, as so-called humates and as sulphates.

Leaching with 4 per cent hydrochloric acid (method A) gave, without exception, more calcium in solution than did method B, and it has been assumed that this increase represented easily decomposable calcium silicates.

Where the CaO equivalent of the CO_2 found was less than the CaO leached by 2 per cent acid, the whole of the CO_2 was calculated to calcium carbonate, but when the CaO equivalent of the CO_2 found was greater than CaO leached by 2 per cent acid, the whole of the CaO in the 2 per cent acid solution was calculated to calcium carbonate.

When the CaO equivalent of the SO_3 found was equal to or less than the water-soluble CaO found, the SO_3 was calculated to $CaSO_4$; but where such equivalent was greater than the water-soluble CaO, the water-soluble CaO was calculated to $CaSO_4$.

The calcium present as difficultly decomposable silicates and that present combined with humus bodies was found by difference, as indicated in the following schematic presentation.

- Total CaO: Determined
  - Not dissolved by 4 per cent HCl: by difference
  - Difficultly decomposable calcium silicates
  - Dissolved by 4 per cent HCl: Determined
    - Not dissolved by 2 per cent HCl: by difference
    - Easily decomposable calcium silicates
    - Dissolved by 2 per cent HCl: Determined
      - Calcium carbonate calculated from CO_2 found
      - Calcium sulphate calculated from SO_3 found in water solution
      - Calcium combined with humus by difference

The figures obtained in this way are presented in Table IV.

TABLE IV.—*Calcium compounds in soils stated as calcium oxid*

CLASS I

Soil.	Total CaO.	CaO as $CaCO_3$.	CaO as $CaSO_4$.	CaO as easily decomposable silicate.	CaO as difficultly decomposable silicate.	CaO with humus.
Holland sandy loam:						
Soil, 0–18 in.	3.18	0.05	.....	0.06	3.01	0.06
Subsoil, 18–40 in.	2.98	.11	Trace...	.09	2.77	.02
Gloucester fine sandy loam:						
Soil, 0–10 in.	2.70	.20	0.01	.10	2.39	.....
Subsoil, 10–36 in.	2.77	.04	.....	.08	2.65	.....
Sassafras loam:						
Soil, 0–9 in	.70	.23	.01	.04	.42	.....
Subsoil, 9–36 in.	.48	.11	Trace...	.02	.34	.01
Collington loam:						
Soil, 0–8 in.	1.00	.14	Trace ..	.05	.66	.15
Subsoil, 8–21 in.	.55	.08	.02	.07	.38	.....
Lower subsoil, 21–36 in.	.63	.05	.01	.04	.53	.....

CLASS II

Spencer silt loam:						
Soil, 0–8 in.	1.26	0.20	Trace...	0.04	1.01	0.01
Subsurface, 8–24 in.	1.11	.08	...do....	.04	.99	.....
Subsoil, 24–36 in.	1.29	.05	...do....	.03	1.15	.06
Lower subsoil, 36–72 in.	.96	.08	...do....	.32	.23	.33
Helmer silt loam:						
Soil, 0–12 in.	1.41	.08	.02	.04	1.27	.....
Subsoil, 12–30 in.	1.34	.13	.01	.05	1.15	.....
Lower subsoil, 30–36 in.	1.26	.12	Trace...	.04	1.10	.....
Helmer loam:						
Soil, 0–6 in.	.57	.05	Trace...	.03	.49	.....
Subsoil, 6–20 in.	1.12	.02	...do....	.02	1.08	.....

TABLE IV.—*Calcium compounds in soils stated as calcium oxid*—Continued

CLASS III

Soil.	Total CaO.	CaO as $CaCO_3$.	CaO as $CaSO_4$.	CaO as easily decomposable silicate.	CaO as difficultly decomposable silicate.	CaO with humus.
Susquehanna clay:						
Soil, 0–5 in	0.98	0.09	Trace	0.23	0.19	0.47
Subsoil, 5–36 in	.96	.15	.....	.39	.10	.32
Cecil clay loam:						
Soil, 0–12 in	.49	.10	Trace	.06	.33	.....
Subsoil, 12–36 in	.59	.04	.....	.03	.52	.....
Colts-Neck loam:						
Soil, 0–6 in	.59	.19	0.01	.03	.33	.03
Subsoil, 6–36 in	.46	.12	.01	.03	.30	.....
Mecklenburg clay loam:						
Soil, 0–4 in	1.98	.12	Trace	.02	1.84	.....
Subsoil, 4–24 in	1.72	.16	...do	.05	1.51	.....
Lower subsoil, 24–36 in	3.88	.14	...do	.23	3.20	.31

CLASS IV

Grundy silt loam:						
Soil, 0–7 in	0.77	0.15	Trace	0.05	0.31	0.26
Subsoil, 7–18 in	.53	.06	...do	.05	.27	.15
Lower subsoil, 18–36 in	1.10	.14	...do	.18	.56	.22
Soil, 0–8 in	1.37	.17	...do	.03	.94	.23
Subsoil, 8–36 in	1.34	.08	...do	.10	.85	.31
Chariton silt loam:						
Soil, 0–7 in	.82	.12	Trace	.02	.56	.12
Subsoil, 7–18 in	.83	.17	...do	.05	.61	.....
Lower subsoil, 18–36 in	.87	.09	...do	.20	.40	.18

CLASS V

Marshall silt loam:						
Soil 0–20 in	1.46	0.18	Trace	0.11	0.97	0.20
Subsoil 20–36 in	1.92	.49	...do	.34	.98	.11
Barnes silty clay loam:						
Soil 0–17 in	1.70	.25	Trace	.09	1.09	.27
Subsoil 17–48 in	6.58	5.67	.02	.03	.86	.....

CLASS VI

Clyde silty clay:						
Soil 0–6 in	1.53	0.23	Trace	0.14	0.58	0.58
Subsoil 6–16 in	1.48	.22	.01	.39	.71	.15
Lower subsoil 16–36 in	1.26	.15	Trace	.23	.59	.29
Soil 0–8 in	1.40	.19	.01	.46	.51	.23
Subsoil 8–12 in	1.29	.13	.01	.20	.51	.44
Lower subsoil 12–36 in	1.14	.07	.....	.37	.33	.37

TABLE IV.—*Calcium compounds in soils stated as calcium oxid*—Continued

CLASS VII

Soil.	Total CaO.	CaO as $CaCO_3$.	CaO as $CaSO_4$.	CaO as easily decomposable silicate.	CaO as difficultly decomposable silicate.	CaO with humus.
Crosby silty clay loam:						
Soil 0–8 in	0.98	0.17	Trace...	0.09	0.61	0.11
Subsoil 8–12 in	.80	.19	.01	.06	.54	.....
Lower subsoil 12–36 in	6.15	5.51	.01	.05	.58	.....
Cherokee silt loam:						
Soil 0–6 in	.60	.07	Trace...	.02	.49	.02
Subsurface 6–16 in	.48	.07	.....	.01	.40	.....
Subsoil 16–28 in	.55	.07	.02	.11	.26	.09
Lower subsoil 28–40 in	.71	.04	.02	.17	.48	.....

CLASS VIII

Vernon very fine sandy loam:						
Soil 0–15 in	0.61	0.14	0.01	0.03	0.41	0.02
Subsoil 15–36 in	.59	.08	Trace...	.03	.30	.18

CLASS IX

Oktibbeha clay:						
Soil 0–4 in	0.65	0.40	Trace...	0.05	0.20	.....
Subsoil 4–36 in	.43	.06	.....	.04	.33	.....

CLASS X

Ritzville silt loam:						
Soil 0–12 in	3.05	0.06	Trace...	0.11	2.58	0.30
Subsoil 12–36 in	5.37	2.50	...do....	.54	2.33	.....
Lower subsoil 36–72 in	5.44	2.72	...do....	.38	2.34	.....

CLASS XI

Iredell clay:						
Soil 0–4 in	4.43	0.10	Trace...	0.07	4.26	.....
Subsoil 4–30 in	4.64	.06	...do....	.11	4.34	0.13

CLASS XII

Pheba fine sandy loam:						
Soil 0–9 in	0.27	Trace.	.....	0.02	0.25	.....
Subsoil 9–36 in	.37	.01	.....	.02	.34	.....

The interpretation of the analytical data in this way is, of course, open to some objections, and all the analytical data have been presented separately in order that any reader may have the opportunity to offer intelligent criticism.

Among the objections to the interpretation just presented the following are probably the most obvious:

CARBONATES.—Where the dilute-acid extract contained CaO equivalent to the CO_2 found, all the CO_2 was calculated to calcium carbonate; but where such equivalent was not found, the CaO in the dilute-acid extract was calculated to calcium carbonate. This is practically an arbitrary decision that in some cases all the CO_2 was derived from calcium carbonate and in other cases it was not. An examination of the figures will show, however, that any error introduced through this assumption is small.

In this connection it should be noted that it is a question whether or not the small quantity of CO_2 found in many instances was really derived from carbonates. It is possible that in some cases these small quantities of CO_2 were derived from the incipient decomposition of some organic constituent brought about by the treatment involved in the method, or that it may simply have been occluded in the soil.

SULPHATES.—The presence of Ca and SO_4 ions in a water extract of a soil is not proof that calcium sulphate existed as such in the soil. This is, of course, true, but gypsum was found in two samples by the petrographic method, and the small quantities stated as calcium sulphate have been presented as an assumption supported only by that fact.

PHOSPHATES.—No provision has been made for calcium phosphates. Apatite was found in one sample by petrographic examination, but it does not seem feasible to attempt a separation of calcium phosphates by analytical methods until some reliable method for distinguishing between organic and inorganic phosphorus is developed.

Calcium phosphates are probably present in many soils, but the quantity must be small and the error introduced by ignoring this in the present scheme also small. Where this error would fall would depend on the character of the phosphate. Apatite would probably appear as difficultly decomposable silicates, and dicalcium hydrogen phosphate probably as calcium combined with humus.

EASILY DECOMPOSABLE SILICATES.—The differences between the quantity of calcium obtained by 4 per cent acid (method A) and that obtained by 2 per cent (method B), stated as easily decomposable silicates, is in some cases very small; such differences as 0.02 and 0.01 per cent are such as would be allowed in duplicates by the same method. This is recognized, and such quantities are not significant, but are stated for uniformity of presentation. It should be noted in this connection that, while these differences are often very small, they are always in the same direction, the quantity of calcium obtained by the more dilute acid always being smaller.

DISCUSSION OF RESULTS

In considering the results of the petrographic examination it should be observed that they are such as have been obtained by a search of a reasonable number of subsamples and the expenditure of a reasonable time. Experience in this work supports the conclusion that by extending the search indefinitely all the minerals reported as not found would have been found in traces.

Any attempt to correlate the petrographic results with those calculated from the analytical data must necessarily be rather unsatisfactory, due largely to the fact already mentioned, that the petrographic method throws no light on the composition of material finer than silt. This gives rise to apparent discrepancies. For instance, samples 58 and 59 showed but a trace of calcite under the microscope, while from the analytical data 2.50 and 2.72 per cent of calcium carbonate was calculated.

In samples 23 and 24 no calcium minerals were found, while the total CaO content was found to be 0.59 and 0.46 per cent.

In general the prevalence of a number of calcium minerals goes with high calcium content as determined by analysis. In samples 3 and 4 five calcium minerals were found, and the total CaO content was 2.70 and 2.77 per cent; in samples 62 and 63 one and two calcium minerals were found in traces only, and the total CaO content was 0.27 and 0.37 per cent.

Hornblende was the most common calcium-containing mineral found, followed in order by plagioclases and epidote. Gypsum, dolomite, and apatite were found but once, and minerals occurring more than once, but of rare occurrence, were usually found in soils from the same location. For instance, augite was found only in samples 57, 58, and 59, garnet in samples 3, 4, 14, and 15, and titanite in samples 3, 4, 15, and 16.

Calcite inclosed in quartz, a form of inclusion very common in some soil types, was found but once, in sample 6. This inclusion of calcite in quartz, of course, introduces an error in the results based on analysis, calcium in this form appearing with the calcium in difficultly decomposable silicates.

In this series of soil samples the total CaO content varies from 0.27 to 6.58 per cent, the CaO as calcium carbonate from a trace to 5.67 per cent. Calcium sulphate, while usually present, does not exceed 0.02 per cent, and CaO in easily decomposable silicates varies from 0.01 to 0.54 per cent.

Except where large quantities of carbonate are present, CaO as difficultly decomposable silicates is the predominating class of calcium compound, varying from 0.10 to 4.34 per cent. Calcium combined with humus compounds varies from 0.01 to 0.58 per cent and is absent from 46 per cent of the samples. This finding is contrary to the common belief that humus compounds in soils are usually combined with cal-

cium and that this calcium compound must be broken up by treatment with a mineral acid before the humus compounds can be extracted with dilute alkali. Experience in extracting a large number of American soils with dilute alkali is, however, quite in keeping with the analytical results here presented, and indicates that there are many soils in which such calcium humus compounds do not occur.

There are four samples with high CaO content in which the calcium carbonate content is also high (samples 39, 48, 58, 59). These are all samples of subsoils of soils derived from material high in calcium carbonate where the carbonate has not been leached from the lower strata.

There are five samples in which the CaO content is above 3 per cent, but where the calcium carbonate content does not exceed 0.14 per cent (samples 1, 27, 57, 60, 61). In these the greater part of the calcium is present as difficultly decomposable silicates.

In considering the calcium-carbonate content of these samples, the question regarding the lower limits of the effervescence test for carbonate arises. All the samples were tested for effervescence by the addition of dilute hydrochloric acid, and no effervescence was observed where the calcium-carbonate content was below 0.40 per cent. The next lowest in the series contains 0.25 per cent, and these results would indicate this test as ordinarily used in the field would be negative where the calcium-carbonate content was less than 0.30 or 0.40 per cent. The data, however, are not sufficient to warrant the fixing of any limit.

Perhaps the most interesting fact brought out by these figures is that it is possible to have two soils with the same or approximately the same calcium content, but containing quite a different assortment of calcium-containing compounds. A few comparisons of this kind have been made and are presented in Table V.

TABLE V.—*Comparison of 12 soil samples containing nearly equal amounts of calcium in various forms*

Sample No.	Total CaO.	CaO as $CaCO_3$.	CaO as easily decomposable silicates.	CaO as difficultly decomposable silicates	CaO with humus.
24	1.98	0.12	0.02	1.84	
36	1.92	49	.34	.98	0.11
19	.98	.09	.23	19	.47
46	.98	.17	.09	.61	.11
14	1.41	.08	.04	1.27	..
43	1.40	.19	.46	.51	.23
10	1.26	.20	.04	1.01	.01
42	1.26	.15	.23	.59	.29
58	5.37	2.50	.54	2.33	..
61	4.64	.06	.11	4.34	.13
18	1.12	.02	02	1.08	..
45	1.14	.07	.37	.33	.37

Other comparisons might be made, but these are sufficient to illustrate the point mentioned and to emphasize the position taken in the introduction that the chemical investigation of soils should not stop with determining their ultimate composition, but should be directed toward ascertaining so far as possible the nature of the compounds in the soil.

It has been well established, with regard to the organic matter in soils, that it is made up of a great variety of compounds and that it is not enough simply to determine the quantity of organic matter or humus, but that research should be extended to the kinds of organic compounds. It is common to find two soils with approximately the same organic content, but containing quite different organic compounds. So it is not inconceivable to have two soils alike in content of calcium, potassium, and phosphorus, and yet, because of the different character of the compounds containing these elements, to be quite different physically, biologically, and agriculturally.

Any discussion of the form of lime in soils naturally should include some reference to soil acidity, and without entering into any discussion of this much-discussed subject, at this time mention will merely be made of some observations on the behavior of the soil samples under discussion toward litmus paper.

The litmus-paper test as an indication of the acid, neutral, or alkaline condition of a soil, in spite of the condemnation expressed in some quarters is still in use by a large number of field observers and laboratory workers who have need for a quick, simple method that will give such indications of a soil's condition as will furnish a basis for advice regarding its treatment or will suggest further investigation. When properly carried out, the authors believe the litmus-paper test affords this. The essentials for the proper performance of the test are good litmus paper and some experience in correlating the results obtained with other observations. It is the experience of the authors that when good litmus paper is pressed in contact with a moist soil and gives a color change indicating an acid condition, a filtered water extract of that soil, on concentration in platinum, will very frequently give a concentrated extract acid to litmus, or will loose volatile acids which by proper means can be recovered and the acid nature demonstrated.

The soil samples included in this investigation were carefully tested with litmus paper, with the result that only samples 49, 50, 51, 62, and 63 were found to be acid. No claim is made that the litmus-paper test as applied to moist soil is at all delicate, and it is quite likely that other samples of this series would be found acid if subjected to a determination of the hydrogen-ion concentration, which is the only true test of reaction. Of those samples found to be acid to litmus, samples 49, 50, and 51 are from one location, and are samples of Cherokee silt loam, 62 and 63 are samples of Pheba fine sandy loam. All are rather low in total calcium content, and in calcium as easily decomposable silicates, and are the lowest of the series in content of calcium carbonate.

It should be noted that both these types are described as having poor drainage, a condition nearly always found when acid soils occur.

Two other soil types represented, Clyde silty clay and Crosby silty clay loam are characterized by poor drainage, but are not acid to litmus. Both are derived from highly calcareous material, and the sample of the lower subsoil of Crosby is really the till from which the soil was derived. The total calcium content of both is fairly high, and the Clyde samples are characterized by a high content of easily decomposable silicates. It is conceivable that easily decomposable silicates might bring about the neutralization of acids being slowly formed in a soil in much the same way that calcium carbonate does.

Another point in connection with soil acidity and the presence of calcium carbonate in soils is the relation of the growing of legumes to these two factors. It seems to be well established that acid conditions are generally unfavorable or even disastrous to such crops, and from this it is assumed that the presence of calcium carbonate is necessary. The data discussed in this paper, while not such as to furnish any basis for a discussion of the subject, are in one particular suggestive. The Mecklenburg clay loam is recognized as a good alfalfa soil. It is high in total calcium content with almost negligible quantities of calcium carbonate.

In conclusion it should be stated that the method by which the results presented have been obtained is put forward as a tentative one and with a full realization of its shortcomings. It is hoped to make this method, or some method of determining the forms of calcium in soils, more comprehensive and less open to objection.

SUMMARY

In this paper there are presented analytical data bearing on the kinds of calcium compounds in 63 samples of soil representing 23 types from 24 locations in 19 States.

From these analytical data the quantities of calcium carbonate, calcium sulphate, calcium combined with humus compounds, and calcium present as easily and difficultly decomposable silicates have been calculated.

The figures obtained show a wide variation in total calcium content and in content of calcium carbonate and the two classes of silicates.

Calcium combined with humus compounds is shown to be absent in 29 samples.

No relation is apparent between the total calcium content and the quantity of any of the classes of calcium compounds discussed.

It is shown that it is possible to have two soils with the same calcium content, but with the kinds of calcium compounds present in quite different quantities.

But five of the samples, representing two types, were acid to litmus. These types are characterized by poor drainage.

A type represented in this series recognized as a good alfalfa soil is characterized by a high calcium content, but low in content of calcium carbonate.

STUDIES UPON THE BLACKLEG DISEASE OF THE POTATO, WITH SPECIAL REFERENCE TO THE RELATIONSHIP OF THE CAUSAL ORGANISMS

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HISTORICAL REVIEW

The fact that the potato (*Solanum tuberosum*) is subject to maladies like that under consideration in this paper was noted at a comparatively early date in the literature of bacterial diseases of plants. De Jubainville and Vesque (8)[1] mentioned in 1878 a "cellular-rot" of potatoes, radishes, carrots, and beets as occurring in the soil and in cellars; but bacteria are not mentioned as the cause. Their paper and the one following are reviewed by Smith (32).

In 1879 Reinke and Berthold (30) pointed out quite clearly that a wetrot of potatoes could occur without the presence of any fungi. According to Smith, they apparently did not have any active parasite and did not work with pure cultures. However, they were able to inoculate and cause a decay of healthy tubers by means of the watery fluid taken from diseased potatoes, provided especially favorable conditions were supplied in the line of moisture. They were also able to demonstrate the constant association of bacteria with the wetrotting of the tubers.

Prillieux and Delacroix (28) described a disease of the potato stem from France in 1890 and gave the name "*Bacillus caulivorus*" to the organism which they considered to be the cause. Nothing is said in this paper as to the isolation of the organism and the growth of it in pure cultures, although later Prillieux (27, v. 1, p. 16) credits it with producing a green coloration of certain culture media. Prunet (29), who reviewed the literature on this subject in 1902, called attention to the fact that Laurent (21) had stated in 1899 that *B. caulivorus* was probably nothing other than *B. fluorescens liquefaciens* Flügge, a common soil saprophyte. Pethybridge and Murphy (26), who have more recently written on the subject, state that—

> Later on Delacroix, in dealing with *B. caulivorus*, speaks of it as most probably identical with *Bacillus fluorescens liquefaciens* Flügge, a common saprophytic form, which, he suggests, may perhaps under certain special conditions become parasitic.

It is evident that Prillieux and Delacroix (28) did not secure the organism responsible for the disease. However, there is every reason to believe that they were concerned with a malady similar to that under

[1] Reference is made by number to "Literature cited," pp. 124–126.

consideration, for it was characterized by a decay of the stem beginning at the base and extending upwards.

It is to Smith (31) that we owe our first tnorough study and careful description of a bacterial disease of the potato stem and tuber. However, the disease, caused by *Bacillus solanacearum* Smith, belongs to a distinctly different type than blackleg.

As far as the writer has been able to discover, Frank, in Germany, (13, 14) appears to be the first writer definitely to connect the blackleg disease or "*Schwarzbeinigkeit*" of the potato stem and the accompanying wetrot of the tuber with a bacterial parasite. It is evident that the disease had been known and recognized there for some time, but it had been associated with various fungi. Frank's description of *Schwarzbeinigkeit* is definite and clear, and it agrees very closely with certain, but not all, of the characters of the blackleg as observed by the writer in Maine, Vermont, New York, and in some of the Middle West and Rocky Mountain States.

The organism, to which the specific name "*Micrococcus phytophthorus*" was applied in the second paper, was found to be constantly associated with the disease and capable of producing it when inoculated into healthy stems. It is interesting to note that all other organisms described as causing a similar type of disease are bacilli and are of greater length than the diameter, 0.5μ, which Frank gave for the cocci he described. In this connection, however, it may be mentioned also that Frank stated that for four years material studied by him from different regions invariably showed organisms of the type he described.

In 1901 Delacroix (9, 11) described what appears to be still another type of bacterial disease of the potato stem for which was suggested the name "*brunissure*," or browning. He gave the name "*Bacillus solanicola*" to the causal organism. As in the case of blackleg, the first visible signs of disease are a yellowing of the foliage and cessation of growth, followed by the gradual death and drying up of the plant. The stems die from the base upward, but are described as browned rather than blackened; and cross sections show transparent brown patches, which may extend some distance up the stem. A prominent characteristic is the development of a gummy material and tyloses in the wood vessels of the stems of attacked plants.

It is evident that this disease, like that caused by *Bacillus solanacearum*, is of a type entirely distinct from blackleg and therefore need not be considered further, especially since cultures of the organism are no longer available for comparison. It may be mentioned, however, that Delacroix considers it to be a soil organism, and the chief source of infection is from the soil by means of wounds. Tubers are also attacked, the disease entering from the stem end and causing a browning of the tissues.

In 1902 Van Hall (15) in Holland described a bacterial disease of the potato which was similar to that described in Germany by Frank. He named the causal organism "*Bacillus atrosepticus*." This will be considered more in detail in the discussion of the organisms studied by the writer.

From 1902 to 1906 Appel published a series of several papers (1–7) upon his studies of the *Schwarzbeinigkeit* as he had observed it in Germany. His most complete account of the disease and the description of the organism was published in 1903 (4). He expressed a view at that time that possibly more than one organism might be associated with the disease and suggested that the term "*Schwarzbeinigkeit*" should not be considered as applying to a specific disease but as descriptive of a pathological symptom. He also mentioned that not all organisms studied by him which were able to cause decay of the tuber were capable of producing the stem disease as well. However, he pointed out that the organism causing *Schwarzbeinigkeit*, which he studied, was probably identical with the one described by Frank. This appears to be the most generally accepted view, although Appel found it to be a bacillus. He published a fairly complete account of it and named it "*Bacillus phytophthorus*." Smith (33) has more recently repeated and extended Appel's studies, working apparently with his original culture, and his description of *B. phytophthorus* is the most complete one that we have.

In a general account of potato diseases and the disease resistance of potatoes, Jones (19), in 1905, describes blackleg and summarizes his observations upon its occurrence in Europe. He mentions it as being found in Germany, Holland, Belgium, France, and England.

In 1906 Delacroix (12) reported on the presence of the *Schwarzbeinigkeit* in France and compared the appearance of plants affected with the latter and those showing "*brunissure*." He states that blackleg is primarily a disease of the late spring or early summer, while the malady caused by *Bacillus solanicola*, as a rule, appears in midsummer or early autumn. However, he says, in effect, that the exterior characters are such that one can hardly separate the two diseases by them.

In the same year Johnson (18) stated that he had clear evidence of the existence of *Bacillus phytophthorus* as a general cause of "yellow-blight," blackleg, and potato tuber-rot in Ireland, but Pethybridge and Murphy (26) are quite inclined to doubt his evidence.

Jones (20) records for the first time the occurrence of blackleg in the United States, also in 1906. The disease agreed in every respect with that which he had observed in Europe. This was in Vermont on a farm where he had been studying potato diseases for nearly 20 years and had not previously observed it. The seed tubers used for planting came from Maine.

In 1906 Harrison (17) described a potato disease of the same type which he stated to be of wide distribution in Canada and of much eco-

nomic importance there. He regarded the causal organism as a new and distinct species to which he gave the name "*Bacillus solanisaprus.*" His study was the most detailed and complete of any which had appeared up to this time upon an organism associated with blackleg.

The writer came to Maine in 1906, too late in the season to find the disease that year, but in 1907 it was found and its presence recorded (22). Preliminary accounts regarding the nature of the disease, economic importance, distribution, manner of dissemination, and means of control were published in 1909 (23) and 1911 (24).

In 1911 Pethybridge and Murphy (26) published a review of the literature on the subject and described a similar type of disease in Ireland. These workers also considered the organism responsible for the type of malady there to possess sufficient distinctive characters to be considered a separate species, although they felt that it was closely related to *Bacillus phytophthorus.* They named it "*Bacillus melanogenes.*"

CHARACTER AND APPEARANCE OF THE DISEASE

Blackleg is, as has already been indicated in the preceding section, a bacterial disease of the stem and tuber of the potato. The various common names which have been applied to it have been given largely as a result of the quite characteristic signs of the disease which are exhibited by the attacked stems. In Germany, where the trouble was first described, it has been known for years as *Schwarzbeinigkeit,* although the term "*Stengelfäule*" has also been applied to it. It would seem from the literature that the former name or a somewhat free translation of it is the one which has been in common use in Holland, Belgium, France, and England. Jones (19, 20) described it under the name "blackleg," the name with which he had become familiar in England and Germany. Smith (33) prefers "basal stemrot." Pethybridge and Murphy (26, p. 9–10) state that—

> Since, however, the term "Blackleg" is one which is already in common use in this country for a disease prevalent among cattle, it seems strongly advisable, in order to avoid confusion, not to use the term "blackleg" for the present disease; and we have, therefore, decided to distinguish it by the name of "Black Stalk-rot."

Were the writer describing this type of disease for the first time, he would endeavor to select a somewhat more appropriate name than blackleg and would prefer black stalkrot or black stemrot to this. However, he has found the objections to the former term to be of more theoretical than real importance.

Blackleg, as the name indicates, is characterized in its typical form by a pronounced blackening of the base of the stem of the affected plant. However, such plants frequently show other visible signs of disease before this character is apparent above ground. They first appear more or less unthrifty and usually undersized. The branches and leaves,

instead of spreading out normally, tend to grow upward, forming a more or less compact top, frequently with the young leaves folded and curled up along the midrib. Later they become lighter green and even yellow, and the whole plant gradually dies. If the disease progresses rapidly, quite a different picture is presented, and the plant may fall over suddenly and wilt with very little previous signs of disease. At first sight the general aspect of the affected plants does not differ from that produced from several other causes which injure or kill the parts at or below the surface of the ground, such as the attacks of fungi or insects, or even mechanical injury at or near the base of the stem. Occasionally, and more frequently some seasons than others, when the plants are attacked before setting tubers and when the progress of the disease is slow, numerous aerial tubers are formed on the stalk at the surface of the ground or in the axils of the leaves above. As is well known, this condition may arise from certain other injuries of the stem as well.

The diagnosis of suspected cases is easily confirmed by pulling up the diseased plants. Stems attacked by blackleg show an inky-black discoloration extending from the base of the stem, where it is attached to the seed piece, up to the surface of the soil, except in the early stages, and very frequently 1, 2, or even 3 inches above the ground. Some seasons, under favorable weather conditions, the disease may with considerable frequency follow up the stem for several inches or even out on the larger branches, destroying the entire stem with great rapidity. This is more likely to occur when the plants are growing in a naturally moist soil and during periods of moist, cloudy weather.

If two or more stalks arise from one seed piece and one develops the disease, the remainder will invariably, sooner or later, succumb to it also. Very frequently during the last of July, or during August in Maine, when a diseased stalk is observed, a careful search will reveal close beside it the dried remains of another which died some time earlier. It is these stalks that are not visibly affected till later in the season which usually show the rapid and complete invasion of the above-ground parts of the stem by the bacteria. However, there is no evidence to indicate that the disease is ever communicated from one stalk to another by traveling down one and up the other. In a large number of inoculations of living potato plants in flowerpots in the greenhouse and in boxes of soil in the field, including all regions of the plant from the petioles of the leaves to parts of the stem below the surface of the soil, the writer has seen little evidence, except in the case of certain inoculations on below-ground parts made by Mr. G. B. Ramsey, the writer's associate, in the summer of 1916, of progress of the disease downward on the stem. It is always upward.

With one exception, the writer has not observed blackleg occurring in patches or localized areas. The attacked plants are scattered promiscuously over the field. The pieces of seed tubers from which such diseased

plants have grown are invariably found to have been attacked by a soft wetrot, or may have been destroyed entirely before the examination is made. At this time and for some weeks thereafter the seed pieces from which surrounding, healthy plants have sprung are entirely sound. If young tubers have formed before the plants are destroyed, the disease frequently passes along the stolons upon which they are produced and infects these also, producing a rapid softrot. This is by no means the invariable rule, as might be inferred from the statements of some writers. However, as will be shown later, it is apparently such cases of infection of the growing tubers which are responsible for the propagation and spread of the disease, either directly or indirectly.

The exception noted above, suggestive of the spread of the disease in the field, occurred in Dover, Me., in 1908, on the same field from which pure cultures of the causal organism were first obtained. There was very little blackleg in the entire field of 20 acres, except on one spot of a few square rods where all of the plants were diseased. It was first noted by the owner near the center of the affected area, from which it gradually spread outward. The season had been excessively wet, and this area coincided with a low, undrained pocket or depression in the field, where water would stand for a few hours after each heavy rainfall.

Sometimes the attacked plants attain a height of only 2 or 3 inches above the surface of the soil, and the fact that many hills are entirely missing on fields which show a high percentage of diseased stems indicates that in such cases either the seed pieces decay before sprouting or the sprouts are killed before they reach the surface. As a rule, however, the plants first begin to show signs of disease when they are 6 or 8 inches high and are growing rapidly. In northern Maine this is about the first of July, although the disease frequently makes its first appearance some time in June. Again, the plants may attain nearly full size before many of them on a field appear to be attacked.

The progress of the disease is markedly influenced by weather conditions. Very moist, cloudy weather during the first few weeks of growth may tend to favor rapid progress, resulting in the early death of the young plants, so that only the dead stalks remain scattered among the healthy plants within a month or six weeks or even a less time after its first appearance. A period of dry weather coming on after the disease is well established below the ground may check its progress but cause the death of the plant at an equally early period on account of its inability to withstand the lessened water supply. Again, conditions between these extremes may prolong the attack well into August. More blackleg is observed in wet than in dry seasons.

Fields which showed quite a percentage of blackleg early in July may give a decidedly different impression to the observer by the middle or last of August. At that time the diseased plants may have entirely disappeared; and on account of the scattered occurrence of the former, the

healthy plants, which have reached practically their full development, obscure the vacant areas, giving the appearance of almost a perfect stand over the entire field.

Soil conditions also are factors which influence outbreaks of blackleg. All other things being equal, the disease is more likely to occur in wet than in dry soil, and is more prevalent when the early part of the growing season is characterized by abundant rainfall.

Different varieties of potatoes show a marked difference in their susceptibility to the disease. This is well shown in the case of the Irish Cobbler and the Green Mountain, which represent by far the greater proportion of the potatoes grown in Maine at the present time. The former is an early variety, largely grown for seed purposes for southern planting, and is highly susceptible. The latter is a late variety which is primarily raised for table use and is seldom severely affected with blackleg. In fact, as long as Maine potato growers planted this variety almost exclusively, blackleg was of minor consequence. Harrison (17) has shown by means of inoculation tests that these differences in ability to resist the attacks of blackleg are exhibited by quite a number of varieties.

It should be understood that the above characterization of the disease is made without reference to the published descriptions of this and similar types of potato-stem disease as they occur elsewhere and is based entirely on personal observations which have been made largely in the State of Maine.

There is a somewhat similar form of potato-stem disease which the writer has seen with some frequency in certain Western States, but which is not common in Maine. It differs from the ordinary type of blackleg in that while the stem is blackened and discolored above ground there is little or no external evidence of disease below ground. In all the cases observed, however, a closer examination has shown that the pith has been destroyed between the base of the stem and the externally diseased area above ground.

A few typical cases of this trouble, which some have been inclined to consider as not identical with blackleg, were seen in Presque Isle, Me., in the summer of 1916. In some instances the disease had spread through the stolons to the young tubers. From one of these tubers Mr. G. B. Ramsey isolated a bacterium which produced typical blackleg when inoculated into young, growing stems.

GEOGRAPHICAL DISTRIBUTION OF THE DISEASE

The fact that blackleg or a very similar appearing disease of the potato stem and tuber has been observed in Germany, France, Belgium, Holland, England, Ireland, Canada, and the United States has been pointed out in the previous section.

From the accounts of Frank (13–14) and Appel (1–7) it is evident that the disease is common, widespread, and often destructive in Ger-

many. Delacroix (12) stated in 1906 that he had not seen it in France until within two years and that it was not widespread at that time. At the same time he pointed out the marked similarity of blackleg to the *brunissure* of the stem previously described by him. The possibility is thus suggested that the malady first described in Germany may be of wider distribution in France than has been reported and that the two have been confused. In the same paper Delacroix says that blackleg exists also in Denmark and Russia.

In writing of the occurrence of blackleg in England, Jones (19, p. 17) stated in 1905 that—

> it is said to be common, though apparently less troublesome than Appel reports it from Germany. Reference to the agricultural papers of England during recent years indicates that the disease is of quite widespread distribution in that country.

Harrison (17), in Canada, reports what is probably a much wider territorial distribution of the type of disease he described than was the case with the previous writers. He states that he had found it throughout the Province of Ontario. Its presence had been reported from Nova Scotia, New Brunswick, and Quebec, and one case had been reported from the Northwest Territory.

In the United States the writer has been collecting information regarding its occurrence during the past nine years. It has been reported in most and doubtless occurs in all of the Atlantic seaboard States from Maine to Texas. It probably occurs also on the Pacific coast, and has been recorded from Ohio, Wisconsin, Minnesota, Colorado, Utah, Montana, and Idaho in the interior. In other words, blackleg has been definitely reported from nearly all the great potato-growing centers or States in this country.

There is reason to believe that Maine was one of the first places in which the disease was introduced into the United States, and in connection with the question of geographical distribution it is of interest to speculate on how this came about. It seems reasonable to believe that it was introduced into Maine from Canada and into the Dominion from England. As will be pointed out in a succeeding section, blackleg is carried by seed tubers. Importation of seed stock into Canada from England would naturally be much more common than into the United States; and the wide distribution of the disease in Canada, reported by Harrison, would indicate that it existed there for some time previous to its entry into Maine. Maine's greatest potato-producing section, where the disease was first discovered, is immediately adjoining the Province of New Brunswick. Many of the potato growers in this region formerly resided on the other side of the boundary line, and with the constant intercourse and traffic between the adjoining sections of the two countries it is readily conceivable that blackleg was imported in this way.

In all probability the disease has been introduced in the same way into other States along the northern border. It was carried to the Southern States by northern seed. There is one authentic instance of the introduction of blackleg into this country directly from England.[1] At Kingston, R. I., in the summer of 1907 five hills of potatoes showing blackleg were found. These potatoes were grown from seed tubers which were obtained from England in the spring of the same year. This was the first and only case of blackleg that had been reported from Rhode Island up to that time.

One interesting and apparent case of importation of the disease from Europe and its transference in this country is as follows: In the summer of 1912 it appeared commonly on a field in Parkman, Me., in a part of the State where the disease was practically unknown, and on a farm where the owner had never observed it before. The field was planted with a variety known as Delmany Challenge, which came from Twin Falls, Idaho. Inquiry showed that this variety had been grown on a farm near Twin Falls for three years, having been shipped there from Carbondale, Colo., where it had been planted for two years. The Carbondale grower imported the original seed tubers from Scotland. The writer has since visited the farms mentioned in Carbondale and Twin Falls and found blackleg present in the potato fields on each.

ECONOMIC ASPECTS OF THE DISEASE

Wherever the type of disease under consideration occurs, those who have described it emphasize its economic importance. Apparently a loss of from 5 to 10 per cent of the plants on affected fields is not an uncommon occurrence in Germany, and much greater losses are reported. Frank (13) reports cases where at least 75 per cent of the plants on a given field were killed from this cause alone. Pethybridge (25) reports the result of experimental trials in which apparently sound tubers taken from an infected crop of the previous season produced 94 per cent of diseased plants.

During the past nine years the writer has had opportunity to make some rather extensive observations on this subject. In Aroostook County, Me., in a fairly continuous area, there are about 50,000 acres of potatoes grown annually. In this region there are few potato fields of less than 10 acres, and usually the acreage of individual farmers runs from 20 to 50, although fields of 60 to 80 are not uncommon and there are those which run to 100 acres or more.

Considerable time was spent in this and other potato-growing sections of the State each year, and during the first five years large areas of potatoes were inspected annually. In many fields only scattered plants have been observed and not infrequently these amount to 1 or 2 per cent,

[1] Reported by Prof. G. E. Adams, of Rhode Island State College, in correspondence with the writer.

5 per cent of diseased plants being considered by the growers as representing a severe attack. However, losses amounting to 10 or 15 per cent or more are by no means unknown; and the writer saw one case in 1911 where 50 per cent of the plants had been killed by blackleg or the potatoes had failed to germinate. It should be mentioned that in this instance the seed tubers apparently had been stored under very adverse conditions and were not in a fit condition to plant. The writer has never seen anything like what Pethybridge has described (25), where apparently sound seed tubers, even if they came from a diseased crop, produced a large percentage of diseased plants. As will be shown in a later section, it has been found that the selection of sound tubers from a diseased crop is an important factor in reducing the amount of blackleg in the following crop.

Much less loss has been observed on high, well-drained soils that were well adapted to growing potatoes than on low, wet, undrained soils. Likewise greater damage has resulted from blackleg in seasons of excessive rainfall than in dry seasons. Much greater losses in Maine apparently have been experienced since the advent of the southern seed-growing industry. As has already been mentioned, certain of the early varieties desired for seed purposes by the growers in the South are more susceptible to the disease. Blackleg also appears to be more destructive in the South than in Maine.

The above discussion has been limited largely to the losses resulting from the failure of the seed tubers to germinate; or if they do germinate, to produce plants which will live to mature a crop. The losses which result from tuber decay either in the field or in storage should also be considered.

Nearly all writers who have discussed blackleg have laid special stress upon the amount of loss which it occasions through destruction of the tubers. Harrison (17) estimates a total loss from rot of from 10 to 75 per cent of the crop in the Province of Ontario. Taking the lower figure and allowing 40 cents per bushel, he states that this would amount to $720,000 in that Province alone.

All of the blackleg-producing organisms described, including those isolated and studied in Maine, are capable of causing a rapid and complete decay of the tubers, especially when they are immature, or immediately following harvesting. There is no doubt that they can and do cause some decay in storage and may be responsible for a considerable amount if storage conditions are unfavorable. The writer is fully aware that the same disease may produce radically different results in different countries under different climatic conditions. However, if he were to base his opinion upon his observations of conditions in the northeastern part of the United States and adjacent portions of Canada during the last 15 or 20 years, he would say that the losses from tuber decay in the field and in storage from this source have been largely overestimated.

It is believed that much of the rot of potato tubers, which in some instances has been attributed to the same organism which produces the blackleg of the stem, is due to entirely different causes. In the Northeastern States epidemics of potato softrot in the field and in storage are quite common, but these invariably follow and are associated with outbreaks of lateblight on the foliage caused by *Phytophthora infestans* De Bary. The decay caused by this fungus, as is well known, is of a dry nature. However, if a tuber lying in a moderately damp soil becomes infected with the fungus, various soil bacteria at once enter into the lesions thus made and a rapid, soft, foul-smelling rot results. Where such decay starts in the field, it is very likely to follow and be very destructive in storage, particularly if the conditions of storage are damp.

Repeated experiments by Jones and his associates in Vermont, Stewart in New York, and Woods and the writer in Maine have demonstrated conclusively that epidemics of such bacterial softrot can be prevented entirely if the parts above ground are kept free from *Phytophthora infestans*, by proper spraying with Bordeaux mixture, until they are ripe or are killed by the frost. Moreover, epidemics of softrot of potato tubers following lateblight were common in New England long before blackleg made its appearance and now occur in sections which are entirely free from blackleg. As has been stated previously, the latter disease has been under observation by the writer to a greater or less extent in Maine each summer since 1907. Two severe epidemics of tuber decay, and other less destructive ones, have been experienced during the same period, but this was entirely controlled where thorough spraying with Bordeaux mixture was practiced. On the other hand, no severe outbreaks of tuber softrot have been observed which were not preceded by lateblight on the foliage, regardless of the amount of blackleg which occurred on the plants in the fields.

It must be conceded, therefore, that ordinarily there is no serious loss from tuber decay from the blackleg organism in Maine. However, it must do some damage of this nature, for there is ample evidence that the infected seed tubers are responsible for the propagation of the disease from year to year. Also it is a natural supposition that conditions which enable the various soil organisms to complete the destruction of the tubers, begun by the lateblight fungus, would also favor decay by the blackleg bacteria. If decay is once started in storage or the tubers are kept under too warm, moist conditions and the latter organisms are present, they certainly would be an important factor in causing the rot. However, the writer has made many attempts to isolate organisms from softrot of potatoes following attacks of lateblight on the foliage; but in no case has he been able to secure a bacterial organism capable of causing the decay independently and alone.

SOURCES OF INFECTION AND MEANS OF DISTRIBUTION

Various other agencies have been suggested as being responsible for the spread of the disease; but a long series of observations have convinced the writer that in Maine the ultimate source of the trouble is, either directly or indirectly, infected seed tubers. A large amount of evidence bearing on this point has been collected during the last nine years, both by observation in Maine and by correspondence with others where Maine potatoes are used for seed. As has already been mentioned, only one case has been observed where the disease appeared to spread in the field; and this is easily explained by peculiar local conditions. Since the organisms, as will be shown later, are killed fairly readily by drying, they are probably incapable of existing for any length of time in a living state on the dry surfaces of potatoes. It is the writer's opinion that they are carried over the winter in decaying, bruised, cracked, or otherwise imperfect seed tubers.

In Maine, potatoes are not stored in covered pits in the ground, as is the case in Ireland and in certain other parts of Europe. They are always kept in dark, cool, well-ventilated cellars or specially constructed potato houses, usually the latter. In these houses considerable attention is paid to ventilation. If possible, no excessive moisture is allowed to develop, and the temperatures are held as low throughout the winter as is compatible with safety to the potatoes. In laboratory experiments the organisms in beef-broth cultures were found alive after a period of 10 months, or until nearly all of the moisture had evaporated from the culture tubes. Temperatures a few degrees above freezing, or in the ice box, have been found to be most favorable for keeping this and similar organisms alive for long periods of time in cultures. Under these conditions the organisms retain their vitality, but multiply at a comparatively slow rate. Therefore it seems probable, if the bacteria are able to enter the interior tissues of the tubers either by natural infection in the field shortly before harvesting, or through wounds or cracks made in harvesting, or even through lesions produced by other parasitic organisms, that, so long as they are supplied with a small amount of moisture, they will remain alive. The low temperatures of storage prevent their rapid multiplication and the resultant decay of the tubers. It is undoubtedly those only slightly affected potatoes which are responsible for the propagation of the disease.

Unfortunately, where blackleg is observed for the first time, it is not always possible to trace the seed tubers to determine whether the disease occurred the season before on the field where they were produced. In all cases where this was possible the answer has been found to be in the affirmative. The only criterion by which to judge matters of this kind is whether or not the characteristic blackening and death of the growing stems occurred. As has already been pointed out, the fact that a large

amount of the previous crop had been lost by decay, whether of the nature of a softrot or not, is not necessarily an indication of the presence of the blackleg disease.

Out of a large number a single case will be cited here as evidence that the disease is first introduced by means of infected seed tubers. In 1907 a 4-acre field on the University farm at Orono, Me., where blackleg had not previously appeared, was planted with seed tubers from several different sources. Along one side three barrels of potatoes, each from a different source, were planted. Quite a percentage of diseased plants were found where one of these barrel lots was used; but a careful search several times during the summer failed to reveal any such on the remainder of the field.

Observations in Maine indicate that under the climatic conditions which exist there infected seed potatoes are the sole source of infection and distribution and that the disease does not live over the winter in the soil. Planting the same field with potatoes two years in succession is quite frequently practiced. Where the disease occurred the first year and sound seed tubers for the second crop were carefully selected and then disinfected with formaldehyde as described in the following section, blackleg was either entirely eliminated or was much reduced, depending upon the thoroughness of the treatment.

On fields which are planted with potatoes the second time in succession there is usually quite a percentage of volunteer plants which spring from tubers which remained in the soil over the winter. These plants are frequently easily recognized by their irregular occurrence on the sides of the rows or between the hills. The writer has never seen such plants affected by blackleg. This observation was quite unexpected, for it seems not unreasonable to suppose that, if the tubers were sufficiently protected from frost to be able to germinate, the bacteria causing the disease might live over in the soil in such infected tubers as well as in storage

CONTROL MEASURES

Laboratory studies showed that the organisms associated with the disease did not form spores, were not resistant to drying, and were readily killed by germicides. This, together with the fact that the disease did not spread from hill to hill in the field and that under Maine climatic conditions decayed, diseased, or otherwise imperfect seed tubers appeared to be the ultimate source of infection, suggested the probability that blackleg might be easily and cheaply controlled or even eliminated from a given farm, field, or locality. The most feasible measures which presented themselves were careful sorting before and at seed cutting, and rigid rejection of all diseased or imperfect seed tubers, especially those which showed any blackened or decayed areas, supplemented as an added precaution by disinfection with formaldehyde or mercuric chlorid.

After certain preliminary experiments and with the realization that no control measures would be of much practical use unless they were effective in the hands of the potato growers themselves, it was decided to ask the cooperation of those interested in the work in order to test these control measures on a large scale under actual field conditions. In 1911, cooperative experiments were conducted by eight practical potato growers in three separate towns. On the eight different farms approximately 300 acres of potatoes were under experiment. Collectively these gentlemen selected and disinfected seed tubers sufficient to plant 142 acres. Formaldehyde solution was used for 88 acres and formaldehyde gas for 54 acres. Detailed statements of the methods used and of the results secured from these experiments have been previously published (24).

The experimental fields were carefully watched by the owners for the appearance of diseased plants. The writer visited some of the farms several times and during the first and third weeks in July made a thorough examination of each experimental field, counting each time the number of diseased plants upon representative areas.

The question might naturally be raised that only two careful counts of diseased plants on each part of the experimental fields during the season would not accurately show the actual amount of blackleg on them, for, as has already been pointed out, the time of the greatest evidence of the disease on a given field may be materially influenced by seasonal climatic conditions. That there may be something to this objection is freely granted; but the writer maintains that it in no way influences the *relative* number of diseased plants on the different plots at the time the counts were made, and this seems to be a fair way of judging the efficiency of the different methods of treatment employed. Moreover, if it were to appear at all on the plots planted at the same time with selected and treated tubers, there seems to be no valid reason why blackleg should not show up equally early in the season as on the check plots planted with unselected and untreated tubers from the same lots or bins. As a matter of fact, the times at which these counts were made were selected because they coincided with the period at which blackleg was most in evidence in surrounding fields that season. Also, as has already been stated, other visits made to the fields at various times during the season and the observations made by the owners of the fields themselves furnish plenty of data to confirm the conclusions derived from the counts of diseased plants.

Taken as a whole, the results of the cooperative experiments were sufficiently clear-cut and conclusive to indicate that the preventive measures outlined are exceedingly efficient if properly carried out. In fact, the uniformity of the results was surprising, since so many individuals, including the men who were employed to cut the seed, were responsible for them. In every case where both carefully selected and treated

seed tubers were used the disease was absolutely eliminated. In two instances this occurred on fields planted the second year in succession, in which considerable blackleg occurred the year before, and also appeared upon the check plots used in the experiment. Also in every case where either disinfection or selection was practiced alone and proper check plots were planted for comparison, the amount of blackleg was materially reduced, except on one field where small inferior tubers were purposely sorted out and planted after first being disinfected with formaldehyde solution.

An analysis of the data furnished by the experiments did not lead to any very definite conclusions as to the relative value of selection of sound, perfect seed potatoes for planting as compared with disinfection with formaldehyde alone. There is no doubt that both are necessary. The writer believes that careful selection of seed tubers and rejection for planting all that are in any way cracked, bruised, discolored, or decayed is absolutely essential, and no amount of disinfection with the present known methods can be relied upon to take the place of it. On the other hand, the formaldehyde treatment appears to be equally essential and must be practiced to supplement selection of seed. No one familiar with bacterial softrots of vegetables would assume or suggest that formaldehyde solution or gas could be relied upon to disinfect entirely or even approximately a tuber the interior of which is partially decayed. The writer has never maintained that this could be done, and it would be unnecessary to refer to it or emphasize the point were it not for the fact that in some instances it has been assumed that he did recommend simply seed disinfection with formaldehyde for the control of the disease.

Unfortunately, the way the experiments worked out there was no opportunity to compare on the same field the relative efficiency of formaldehyde gas and solution. In every case where formaldehyde gas was used the results were less efficient than those where formaldehyde solution was used. For this and other reasons the gas treatment is not recommended for general use.

Numerous other cases have come to the writer's attention where careful selection of sound, healthy seed tubers, followed by disinfection with formaldehyde has either eliminated or largely reduced the amount of blackleg in the resulting crop, but only one of these will be mentioned.

In 1911 the Maine Agricultural Experiment Station purchased two lots of Irish Cobbler potatoes for planting at Highmoor Farm in the central part of the State. These were disinfected with formaldehyde, but no special care was used in selection before planting. In both cases quite a percentage of the resulting plants were attacked by blackleg. The following season the farm superintendent personally saw that the writer's recommendations relative to seed selection and disinfection were rigidly carried out. A clean crop resulted, and since then no blackleg has been observed on this farm.

In 1913 some of the crop raised from these selected and disinfected seed tubers were sent to Director T. C. Johnson, of the Virginia Truck Experiment Station, for planting at Norfolk. Out of over 3,000 plants produced from this seed only two doubtful cases of blackleg were reported. At the same time two lots of seed tubers purchased in the open market gave 6.5 and 7.9 per cent, respectively, of diseased plants, and another lot sent in for testing gave 16.8 per cent.

Undoubtedly much can be gained by uprooting and destroying all blackleg plants in the field as soon as seen, taking care to remove also all tubers which have formed; but the writer has no experimental data bearing on this point. It should be recommended not as a substitute for seed selection and disinfection, but rather to supplement them, for the latter are, of course, equally important in the control of several other diseases which are carried by the tubers.

COMPARATIVE STUDIES OF THE CAUSAL ORGANISMS

Of the various organisms which have been isolated and described in different parts of Europe and in Canada as being the cause of *Schwarzbeinigkeit*, or blackleg of the stem of the potato, and the attendant decay of the tubers, the following are available for study: *Bacillus atroscpticus* Van Hall, *B. phytophthorus* Appel, *B. solanisaprus* Harrison, and *B. melanogenes* Pethybridge and Murphy. As far as could be learned from reading the published articles and descriptions, the different investigators who named the species in question made no comparative, cultural studies in the laboratory, under identical conditions, of the previously described species and the organisms which they themselves had isolated. They based their conclusions with regard to the nonidentity of the species upon the manner in which they found their own organisms to differ from the descriptions previously published.

Therefore, when the writer began his studies of this disease in Maine in 1907, several interesting questions presented themselves. From both a scientific and a practical point of view the most important question was, Is the disease in the United States, especially in Maine, caused by one or more of the named species of blackleg-producing bacteria or by one differing from each of them in certain well-defined characters? In other words, if the previously described organisms were collected together and subjected to the various differential tests usually employed in describing a species of bacteria under exactly identical laboratory conditions, would these differences still hold or would they all prove to be identical with each other and with those found associated with the disease in Maine? The exerience of the writer (16) in a similar study of the very closely related group of bacteria causing a softrot of various vegetables suggested that the last question possibly might be answered in the affirmative. Accordingly, after considerable preliminary study had been made of some 18 different strains of organisms isolated in different parts of

Maine, in order to show that these were probably identical with each other and very closely related to *B. solanisaprus* three of these representing isolations made from diseased plants from widely different parts of the State were selected for detailed study and an attempt was made to obtain all of the available named species for comparison. The names of the organisms thus secured and the sources from which they came are given in a later section.

While the work was in progress, Smith (33) published his report on his studies of *B. phytophthorus*, mentioned below. It should be noted here that while he did not at this time publish in detail on his findings with regard to *B. solanisaprus* he states that: "*Bacillus solanisaprus* Harrison is a very closely related, but not identical organism, causing a similar disease in potatoes." In a letter to the writer, about two weeks before this paper was read, dated December 18, 1909, he stated that he had for three years been making a comparative study of *B. phytophthorus*, *B. solanisaprus*, and *B. solanacearum*. It is evident then that the sentence quoted above was based upon a series of long and careful comparative studies. Therefore the writer is warranted in the beginning in assuming that, while very closely related, there are in the opinion of Smith at least two separate types or species to be considered.

For the purpose of comparison it seems necessary to state as briefly as possible, without sacrificing accuracy of statement, the descriptions of the four previously named species which have been included in the present comparative study with the organisms isolated in Maine by the writer. For convenience, since Smith's description of *B. phytophthorus* (33) is more recent than Appel's, and since Harrison's (17) is the first extended and most complete description of the type of the organism that the writer has found in Maine, these will first be given in detail, followed by a brief statement of the characters by which the descriptions of *B. atrosepticus* and *B. melanogenes* would appear to differentiate the last two named from each of the first and from each other.

DESCRIPTION OF ORGANISMS PREVIOUSLY DESCRIBED

SMITH'S DESCRIPTION OF BACILLUS PHYTOPHTHORUS APPEL [1]

The organism is a non-sporiferous rod, variable in length, usually occurring singly or in pairs, but also forming chains of several individuals; taken from young agar cultures the diameter is about 0.6 to 0.8μ, the length 1.5 to 2.5μ; actively motile by means of peritrichiate flagella; stains readily with ordinary stains, but not by Gram's method, rots potatoes (stems and tubers), cucumbers, tomatoes, etc.; aerobe and facultative anaerobe; organism grayish white on agar and slightly bluish opalescent by transmitted light; surface colonies, on thinly sown +15 agar, 1 mm. or less in diameter in 48 hours at 20° to 23° C., 2 to 3 mm. broad in 4 days; round, smooth, wet-shining, internally reticulated at first, amorphous under 16 mm. and 12 ocular, or with small flocks in the older portion; the buried colonies appear brownish under the microscope, also

[1] Quotation 748 from a paper read at the meeting of the American Phytopathological Society, Boston, Dec. 30 and 31, 1909. (33.)

granular in the center; margin of buried colonies sharply defined; liquefaction of +10 gelatin moderate to rapid; circular white colonies with regular margins on gelatin plates, visible in 18 hours at 30° C., in 26 hours at 21° to 23° C.; on thin-sown gelatin plates colonies grow rapidly and are frequently 2 centimeters in diameter at the end of fourth day at 22° C.; alkaline reaction in gelatin cultures to which litmus has been added; on sterilized potato slow white to yellowish white growth; characteristic rapid white growth and black stain on raw potato (when streaked from agar); grows vigorously and with great rapidity on all neutral and feebly alkaline media; clouds 10 cc. of +15 bouillon in 6 hours at 30° C. and in 24 hours at 13° to 14° C., when inoculated with one 3-mm. loop from a bouillon culture 4 days old at 24° C.; especially good growth on neutralized potato-juice gelatin in which stab-cultures rapidly develop a funnel-shaped liquefaction, but less rapid in my hands than in +10 peptonized beef-gelatin; gradual clouding of salted peptonized beef-bouillon, and production of chains therein and pellicle on undisturbed old cultures; no indol reaction; tolerates in beef-bouillon a considerable amount of sodium chloride (5 per cent) and of sodium hydrate (+50); very active growth in potato-juice with formation of thick pellicle and heavy precipitate; rapid clouding of closed end of fermentation-tubes containing potato-juice, but no production of gas; no growth in Cohn's solution; slight greenish tinge in Fermi's solution on long standing; moderate production of hydrogen sulphide; distinct and persistent nitrite reaction in nitrate bouillon but no gas; grows in peptonized beef-bouillon from −50 to +16 and beyond, also in potato broth acidulated to +46 with citric acid, but no growth when acidulated to +45 with oxalic acid; slow (acid) coagulation of milk with precipitation of the casein; slight reddening and final reduction of litmus in milk; slight production of gas in shake-cultures in some beef-agars; grows in bouillon over chloroform; in streak-cultures it reddens litmus agar decidedly in 48 hours at 20° C. in presence of either dextrose, saccharose, lactose, gelactose or maltose; it blues plain litmus agar decidedly in 48 hours and does not promptly redden the same with addition of dextrine or glycerine; no reddening of litmus in gelatin-cultures; the acid persists on boiling; produces small quantities of gas from innosit (muscle sugar), lactose and mannit; optimum temperature 28° to 30° C.; little growth below 4° to 5° C.; minimum temperature for growth in +15 beef bouillon 1° C. or under; maximum temperature for growth in +15 beef-bouillon about 36° C.; thermal death-point in +15 beef-bouillon 47° C.; 90 per cent destroyed by freezing in bouillon. Appel reports loss of virulence in some of his cultures, but I have not observed any during a period of three years. * * * The following are recommended as quick tests for differential purposes: Very thin sowings on gelatin plates; streaks from agar to sterile raw potato; behavior in blue litmus milk; behavior in nitrate bouillon and in Cohn's solution. The right organism should produce big, round, white colonies promptly on thin sown gelatin plates, and should rot potato tubers promptly.

BACILLUS SOLANISAPRUS HARRISON

The following is a brief of Harrison's original description of this organism (17). No effort has been made to follow the original form, but care has been taken to preserve accuracy of statement:

Bacillus, with slightly rounded ends, variable in size, according to the media and temperature in which it was grown. From freshly infected potato stems and tubers it varies in size from 1.5 to 4μ long and about 0.6 to 0.9μ wide. From beef-peptone-agar cultures 24 hours old at 20° C. the bacteria were short and stout, rather variable in length, ends rather square but rounded, vacuolated with average dimensions 1.2 by 0.6μ. From beef-peptone gelatin +5, 24 hours old at 20° C. the bacilli were short and stout, ends slightly rounded, some vacuolated, dimensions 1 to 2.5 by 0.6 to 0.8 μ. Involution forms, long swollen bent rods in 24 hours at 37° C.; endospores

not seen; flagella, 5 to 15 or more, peritrichously disposed; stained by Van Ermengem's method; organism stained readily with the usual anilin colors; Gram's method was negative, but positive with amyl alcohol; growth in agar abundant, filiform, spreading, elevation raised, luster glistening, opalescent; good persistent growth on potatoes, slightly raised and spreading, dull waxy and pale cream in color which subsequently changed to dirty white; growth in Loeffler's blood serum good and slightly spreading, raised a little with the edges some higher than the center, waxy and pale yellow in color; uniform growth in gelatin stab cultures, filiform, liquefaction begins in 35 days and is not complete in 44 days; strong growth in nutrient broth +15 at 25° C., slower at 20° C.; ring in surface growth; clouding strong and persistent, fluid turbid, fine sediment; prompt coagulation of milk in 48 hours, extrusion of whey in 3 days, a few minute bubbles of gas visible; litmus milk acid, with partial slow reduction; growth in gelatin colonies slow, round to elliptical or egg-shaped in form; elevation, surface colonies flat; edge first entire, later lobate or erose; color faint brown by reflected, and bluish white by transmitted light; agar colonies round to lenticular, surface moist, shiny; elevation flat; edge entire; internal structure finely granular; growth in Fermi's solution similar to that in Uschinsky's medium, but slightly less in amount; copious growth in Uschinsky's solution, ring, sediment, liquid appeared bluish; the best media for long continued growth was found to be beef bouillon and Uschinsky's; in fermentation tubes gas was produced only in bouillon containing mannit and lactose; growth in the closed arm with production of acid in the presence of each of the following: Dextrose, saccharose, lactose, maltose, glycerin, mannit, and levulose; at the end of 10 days the closed arm with mannit, glucose, and lactose was clear, the other tubes remained clouded in the closed arm, but with less turbidity than was observed on the second day of growth, nitrates in nitrate broth were reduced, nitrites present; indol production moderate to feeble; growth in bouillon +16, Fuller's scale, with hydrochloric acid and not at +18; growth in bouillon −16 with NaOH but not at −18; vitality on culture media, long; thermal death point 54° C., the optimum temperature 25° to 28°, slight growth at 37°, none at 42°; maximum temperature for growth 37.5°; minimum temperature for growth about 0°; in poured plates sensitive to sunlight, 90 per cent being killed in 30 minutes and 100 per cent being killed in 50 minutes; pathogenicity proven in the following vegetables: Potato, tomato, Jerusalem artichoke, cucumber, red carrot, white carrot, radish, red beet (sl.), sugar beet (sl.), parsnip, cauliflower, cabbage, celery, mangel-wurzel, Swede turnip, and white turnip; also in living plants of potato, tomato, common red pepper, and slightly in cucumber and physalis.

BACILLUS ATROSEPTICUS VAN HALL

The description of this species was published in Dutch (15) as a part of a dissertation for the doctor's degree. The following is based on a translation of a part of this article, made for the writer by Dr. R. de Zeeuw.

Bacillus occurring in 2-day-old bouillon cultures at 27° C., almost exclusively as single rods, sometimes in pairs; size variable, 0.8 to 1.6μ long by 0.2 to 0.4μ wide, stained with gentian violet; many small zoogloœa; very motile at 27° C. in young cultures containing 0.025 per cent of potassium phosphate and 0 25 per cent asparagin; flagella stained from such cultures by Loeffler's method, length 10 to 15μ; Gram's stain decolorized. Growth weak on malt agar and malt gelatin; gelatin liquified, but rapidity is variable, does not take place on unneutralized meat gelatin and is sometimes slight on one weakly alkaline. Strong coagulation of casein in milk. No diastasic action on starch. Gas production weak or absent except where mannit is used as a source of carbon. A medium consisting of "duinwater" (water out of the dunes, filtered through sand and gravel) plus 0.025 per cent of dibasic potassium phos-

phate (K_2HPO_4), 1 per cent peptone, and 3 per cent, respectively, of different carbohydrates gave the following results in three successive trials: Saccharose, 0.1, 0, 0.2; glucose, 0, 0, 0.1; lactose, 0, 0, 0; mannit, 0.4, 0.6, 0.3; glycerin, 0, 0, 0; galactose not changed; nitrates reduced to nitrites; reduction of methylene blue weak; sodium selenite reduced very rapidly; indol production not observed. Growth in bouillon acidulated till the reaction is 0.5 per cent normal with citric and malic acids does not stop growth, but 1 per cent entirely prevents it. Thermal death point for a 24-hour-old culture between 51° and 52° C. Optimum temperature not accurately determined, but strong growth takes place at 27° C. Readily killed by drying.[1] Pathogenicity to potato stems and tubers somewhat variable, but apparently imperfectly tested.

BACILLUS MELANOGENES PETHYBRIDGE AND MURPHY

Pethybridge and Murphy described their organism in considerable detail (26), but it is sufficient for our present purpose to simply state wherein they found it to disagree with published descriptions of previously described organisms. They stated that it was larger in size than *B. atrosepticus* (0.7 to 0.9 by 1.3 to 1.8μ) and instead of occurring chiefly singly was found more frequently in pairs, also that its action upon milk appeared to be different. As contrasted with Harrison's description of *B. solanisaprus*, it possessed less flagella, formed gas in glucose and cane sugar, did not form a distinct ring on the surface of potato juice, and did not produce a raised, creamy white growth on cooked potato. They stated that their organism showed marked resemblance to *B. phytophthorus* and that they were strongly tempted to regard it as only a variety of the latter. However, it did not produce a pellicle on Appel's sterile potato juice, and in nitrate broth it produced a small quantity of gas. In milk it caused the separation of the curd as a not very compact mass and produced a distinct acidity in a comparatively short time. According to Appel's description, *B. phytophthorus* produces a strong pellicle on sterile potato juice, apparently produces no gas in nitrate broth, and causes milk to change only on long standing, forming then a compact cylinder of precipitated curd, and giving a reaction which is amphoteric to litmus.

SOURCES OF THE CULTURES USED

Blackleg was first observed by the writer in Maine at Sherman, on July 30, 1907. It was seen later at Orono and Dover; but in all cases the disease was in its last stages, and attempts to isolate the causal organism that season resulted in failure.

In 1908 diseased plants were obtained from Dover, Piscataquis County, on July 24, which showed the affected tissues filled with actively motile bacteria. Several subcultures were made from plates poured from these stems and all proved to be nonpathogenic.

At the same time the diseased portions of the stalks were mashed up with distilled water in a mortar. Healthy potato tubers, bearing shoots

[1] The author also presents considerable data relative to experiments conducted to determine the available sources of nitrogen and carbon, using a considerable number of different substances, apparently added to the culture medium singly or in combination.

2 or 3 inches long, were thoroughly moistened with this watery extract of the diseased tissues and at once planted in boxes of soil which had previously been thoroughly wet down. A part of the remaining watery extract was then poured over each box. These artificially infected tubers put up shoots very rapidly and on August 15, 22 days after the planting, one stalk began to show the characteristic signs of the disease. On the 18th two others were also plainly affected. Examination showed only motile, rod-shaped organisms in the diseased tissues. Poured plates were made from these stems and 9 subcultures were obtained, all of which proved to be pathogenic to potato tubers and stems, producing typical blackleg when inoculated into the latter. Some preliminary work, especially in the line of fermentation studies, was done with all of these, but one strain, designated as "IIIA," was selected for detailed study.

On August 12, 1908, a potato stem about 18 inches long was received from the University Farm, Orono, Penobscot County, which showed a soft, colorless decay which apparently was progressing very rapidly. The general appearance was decidedly different from the picture presented in typical cases of blackleg. The stem was affected nearly its entire length and there were no signs of blackening. The disease was confined almost entirely to the parenchyma cells of the pith within the vascular ring. Apparently the water conducting system had not been materially affected, as there was no yellowing or other abnormalities of the foliage and very little signs of wilting, although one or two other stalks in the same hill had begun to fall over.

The affected portion had been reduced to a pulpy, watery mass; apparently the middle lamella had been destroyed between the cells which latter had collapsed into irregular, shapeless masses. The liquid between the cells was filled with large numbers of motile bacteria. From plates made from this stem 5 subcultures were obtained, all of which proved to be pathogenic to potato tubers and stems. However, in all inoculation tests extending over a period of eight years, typical cases of blackleg were produced, differing materially from the appearance of the original stem. One strain, marked "SE," was studied as a representative of this type.

In August, 1910, another series of four cultures, of which "IIP" was selected as a representative, were isolated from a typical blackleg stem received from Presque Isle, Aroostook County.

Thus, it will be seen that the organisms which were used for the detailed studies were obtained from somewhat widely separated localities, representing two typical cases of blackleg and a third which looked like a radically different type of stem disease, characterized by a more rapid and much more extensive decay of the stem without the development of any blackening or discoloration. As will be seen later, these proved to be, except for slight variations in size, all of the same type as *B. solanisaprus* which was not unexpected in view of the fact that Harrison has found this organism of wide distribution in Canada and there is considerable reason

to believe that the disease was introduced into Maine from Canada. It should be noted, however, that Smith (33) reports having isolated *B. phytophthorus*, which he considers to be closely related to but not identical with *B. solanisaprus*, from potatoes grown in Maine.

At the beginning of these comparative studies in 1908 an attempt was made to collect cultures of all the available organisms which had been described as causing a similar type of disease upon potato stems and tubers.

So far as could be learned, *B. atrosepticus* Van Hall was the earliest described species available. A culture so named was finally obtained from Král's Bacteriologisches Laboratorium, Prague, Austria.

Difficulty was experienced in obtaining cultures of *B. phytophthorus*. The cultures were requested of Dr. E. F. Smith, of the United States Department of Agriculture, and of Dr. O. Appel, of the Biologische-Anstalt, Dahlem. Dr. Smith, while expressing a willingness to pass along the culture, stated that he preferred not to part with it till he had published on the subject. Two cultures bearing this name were received from Dr. Appel. These were apparently identical. Neither of them proved to be pathogenic, either to potato tubers or stems, on repeated inoculations. Apparently Dr. Appel had lost his original culture, from which, presumably, Dr. Smith's strain was obtained, and writing under date of February 14, 1910, he stated that it had been necessary to make a fresh isolation of the organism before he could send it. The writer again wrote to Berlin in the summer of 1911, and a second culture was sent him. This reached the writer in February, 1912, by hand of Dr. W. A. Orton, of the United States Department of Agriculture, who kindly consented to bring it from Germany. Accompanying the culture was the following statement:

> *B. phytophthorus* is isolated from potato tubers by Dr. Schuster, 1911, at Dahlem, Berlin, whose paper on rot of potato tubers is going to be published.

This culture was also found to be nonpathogenic to potato stems and tubers, and, as will be shown later, showed certain well-marked differences in its behavior on culture media when grown side by side with the culture received earlier from Dr. Appel under the same name. It should be remarked here that Dr. Appel recorded the fact that with him some cultures of *B. phytophthorus* showed a less virulence after growing for some time on artificial media. On the other hand, Dr. Smith (33, p. 749) states: "I have not observed any during a period of three years." None of the other organisms studied by the writer have shown any loss of virulence on long-continued cultures, stock cultures being carried either in potato broth or beef-extract bouillon.

My request to Dr. Harrison for a culture of *B. solanisaprus* was referred to Prof. S. F. Edwards, then of the Ontario Agricultural College, who furnished me with a virulent culture in March, 1909.

B. melanogenes was received from Dr. Pethybridge in 1911. This has also shown a good degree of virulence and like *B. solanisaprus* has repeatedly produced on inoculation from pure cultures a rapid and complete softrot of potato tubers and a typical decay of the stem resembling in every particular the blackleg as it occurs in the field.

METHODS USED

In the present investigation the writer has endeavored to follow as closely as possible the procedures outlined by the Committee on Standard Methods of Water Analyses [1] and those recommended by Smith (32, v. 1).

For what seemed good and sufficient reasons, certain deviations were made from the procedures recommended above; but wherever this has been done, mention has been made of the fact and each named organism or cultural strain has been treated exactly like all others. The most important change has been the substitution of Liebig's extract of meat for infusion of lean beef as a basis for ordinary bouillon, gelatin, agar, and bouillon containing carbohydrates for fermentation purposes.

The reasons for this are that the meat extract is a standard product, compounded on a large scale by a reliable concern, and can be purchased the world over, while, on the other hand, no two lots of lean beef obtained from the market have exactly the same composition with regard to amount of fat or fiber present, or decomposition products developed from the different periods of storage before use. The chief objection to the use of lean beef as a basis of fermentation broths is that it is first necessary to remove the muscle sugar from the meat infusion by inoculation with *Bacillus coli*. Consequently the test for fermentation which follows is made, not in a culture medium most favorable to the growth of the organism to be tested, but in one containing the various metabolic by-products of another. Each lot of meat-extract bouillon before being used for fermentation purposes was first tested for fermentable carbohydrates by filling a fermentation tube with it and inoculating it with *B. coli*. In no case was any gas produced. Moreover, in many tests of bouillon made from Liebig's extract during the last 15 years, in only one instance did any gas develop and then only a small bubble in the end of a fermentation tube, the closed arm of which contained approximately 25 c. c. of medium.

However, a careful comparison was made as to the rate of development and vigor of growth of the various organisms in bouillon made from an infusion of lean beef, and in that made from Liebig's extract. In ordinary bouillon no difference was observed between the two in this respect. In fermentation broth made from the meat extract a better

[1] Report of Committee on Standard Methods of Water Analysis to the laboratory section of the American Public Health Association, presented at the Havana meeting, Jan. 9, 1905. 141 p. Chicago, Ill., 1905. Reprinted from Jour. Infect. Diseases, Suppl. 1, 1905.

growth and more constant results were obtained than with that made from lean beef and freed from fermentable carbohydrates by use of *B. coli*.

All chemicals employed in making media or testing reactions were either chemically pure or were of the highest purity obtainable. In addition to Liebig's extract, Witte's peptone, "Gold label" gelatin or Nelson's photographic gelatin No. 1 were used, the latter entirely during the last of the work. For bouillon 5 gm. of Liebig's meat extract and 10 gm. of Witte's peptone were used for each liter of distilled water. To this were added 15 gm. of agar shreds or flour, or 100 gm. of gelatin if a solid medium was desired. No sodium chlorid other than that contained in the meat extract was added. All media unless otherwise specified were made neutral to phenolphthalein. Distilled water was used for all culture media and Grübler's dry stains formed the basis for all staining liquids.

Tubes of ordinary bouillon and agar were sterilized by heating once in an autoclave for 15 minutes at from 3 to 6 or 7 pounds' pressure. All vegetable media or media made from vegetables or containing sugars were sterilized by flowing steam, for tubes, 15 to 20 minutes at from 99° to 100° C. on each of three consecutive days. In the earlier part of the work gelatin was also sterilized by the discontinuous steaming method. Later it was found that it was less likely to become liquid at ordinary room temperatures if the tubes were sterilized by one exposure in the autoclave to steam under 5 to 7 pounds' pressure for 15 minutes.

At the beginning of the comparative work with the assembled cultures each was transferred to a fresh potato-broth culture every 24 hours for several successive days and then gelatin plates were poured from the last potato-broth culture. From these plates transfers were made from single colonies to tubes of sterile bouillon to furnish fresh subcultures with which to work, after proving that the subcultures thus obtained were pathogenic. This was to insure that the cultures were in as near a uniform condition of vigor as possible before beginning to work. Attempts were made to revive in this way the pathogenicity of the two strains which were received as *B. phytophthorus*, but without success. A separate series of stock cultures of each strain which had not been put through this revivifying process were kept in reserve.

All transfers to media to test cultural features were made from young broth cultures about 48 hours old, when the cloudiness had reached its maximum density. For stab cultures to agar and gelatin a straight platinum needle of approximately 0.5 mm. in diameter was used. All transfers to liquid media were made with a 2-mm. loop of the same sized wire. Unless otherwise specified the cultures were incubated at 20° C. In making records of the detailed features of the morphology, cultural, physical, and biochemical features, etc., of the various organisms the descriptive chart or card adopted by the Society of American Bacteriologists in December, 1907, was used as a model and guide.

DETAILS OF COMPARATIVE STUDIES

MORPHOLOGY

B. atrosepticus, *B. solanisaprus*, *B. melanogenes*, and the three organisms studied which were isolated from Maine potato stems were found to agree in all essential morphological characters except for certain variations in size. They are motile, rod-shaped organisms with peritrichiate flagella, approximately three times as long as broad, occurring singly and in pairs, and in unstained preparations from young cultures often showing chains of several individuals. Frequently, however, rather thick rods with very little difference in the two dimensions were observed, more especially in preparations made from the decayed tissues of inoculated tubers. The organism obtained from Dr. Appel as *B. phytophthorus*, while agreeing in other morphological characters, was distinctly smaller in size and the ratio between length and width was decidedly less. No evidence of spore formation was obtained. Involution forms were not observed, although Harrison (17) reports them for *B. solanisaprus* when grown at 37° C. In this connection it may be mentioned, as is stated later, that at this temperature the writer has been unable to secure visible growth of any of the pathogenic strains either on agar slants or in tubes of beef broth.

For making measurements all of the different strains studied, except that obtained from Dr. Schuster as *B. phytophthorus* (which was a nonpathogenic organism plainly of an entirely different type), were stained with aqueous gentian violet, anilin water gentian violet, aqueous methylene blue, alkaline methylene blue, aqueous fuchsin, and carbol fuchsin. These preparations were made from agar slants 36 to 48 hours old. Flagella were stained by a modification of the Pitfield method.[1]

The results of these measurements are given in tabular form below. Table I gives for each organism the extremes in length and breadth and the average size obtained as shown by all the stains collectively. The second presents the same data for each organism for each separate stain used.

TABLE I.—*Extreme and average measurements (in microns) of each organism as obtained from using six different stains*

Organism.	Width.	Length.	Average size.
B. atrosepticus	0.4 to 0.8	1.3 to 2.5	0.5 by 1.8
B. solanisaprus	.4 to .7	1.0 to 2.2	.5 by 1.4
B. melanogenes	.4 to .8	1.2 to 2.4	.5 by 1.7
IIIA	.5 to 1.1	1.3 to 3.3	.7 by 2
SE	.4 to .8	1.3 to 2.5	.6 by 2
IIP	.4 to .8	1.3 to 2.9	.6 by 1.9
B. phytophthorus from Appel	.3 to .7	.5 to 1.6	.5 by .9

[1] The writer wishes to acknowledge the aid rendered by Messrs. M. Shapovalov and A. Strauss in assisting in making many of these preparations, particularly the laborious task performed by the latter in making the large number of measurements of stained organisms, which is the basis of the data here presented.

TABLE II.—*Measurements (in microns) of each organism arranged according to method of staining*

AQUEOUS GENTIAN VIOLET

Organism.	Width.	Length.	Average size.
B. atrosepticus	0.5 to 0.7	1.6 to 2.5	0.6 by 2
B. solanisaprus	.4 to .7	1 to 1.9	.5 by 1.3
B. melanogenes	.4 to .7	1.3 to 2	.5 by 1.6
IIIA	.5 to .7	1.6 to 2.8	.6 by 2.1
SE	.5 to .8	1.6 to 2.5	.6 by 2
IIP	.4 to .7	1.5 to 2.6	.6 by 1.9
B. phytophthorus from Appel	.3 to .6	.6 to 1.4	.5 by .9

ANILIN WATER GENTIAN VIOLET

B. atrosepticus	0.4 to 0.7	1.3 to 2	0.5 by 1.6
B. solanisaprus	.4 to .5	1.0 to 1.6	.5 by 1.2
B. melanogenes	.4 to .7	1.3 to 2.1	.5 by 1.7
IIIA	.5 to .8	1.5 to 2.5	.6 by 2
SE	.5 to .8	1.6 to 2.5	.6 by 2
IIP	.5 to .7	1.4 to 2.9	.6 by 1.9
B. phytophthorus from Appel	.4 to .6	.5 to 1.6	.5 by 1

AQUEOUS METHYLENE BLUE

B. atrosepticus	0.4 to 0.8	1.3 to 2.3	0.5 by 1.8
B. solanisaprus	.4 to .6	1 to 1.9	.5 by 1.4
B. melanogenes	.4 to .7	1.3 to 2	.5 by 1.7
IIIA	.5 to 1	1.7 to 3.3	.6 by 2.3
SE	.5 to .8	1.6 to 2.5	.7 by 2.1
IIP	.4 to .8	1.6 to 2.9	.6 by 2
B. phytophthorus from Appel	.3 to .6	.7 to 1.3	.5 by .9

ALKALINE METHYLENE BLUE

B. atrosepticus	0.4 to 0.7	1.4 to 2.3	0.5 by 1.8
B. solanisaprus	.4 to .7	1 to 2.2	.5 by 1.5
B. melanogenes	.4 to .8	1.3 to 2.4	.5 by 1.9
IIIA	.5 to 1	1.7 to 2.9	.8 by 2.2
SE	.5 to .8	1.5 to 2.5	.6 by 2
IIP	.4 to .8	1.5 to 2.8	.6 by 2
B. phytophthorus from Appel	.4 to .7	.7 to 1.4	.5 by .9

AQUEOUS FUCHSIN

B. atrosepticus	0.4 to 0.7	1.3 to 2.3	0.5 by 1.8
B. solanisaprus	.4 to .7	1 to 2	.5 by 1.4
B. melanogenes	.4 to .7	1.3 to 2	.5 by 1.6
IIIA	.5 to .8	1.6 to 2.5	.7 by 2
SE	.4 to .7	1.5 to 2.3	.6 by 1.9
IIP	.4 to .7	1.3 to 2.5	.5 by 1.8
B. phytophthorus from Appel	.3 to .6	.5 to 1.3	.5 by .9

TABLE II.—*Measurements (in microns) of each organism arranged according to method of staining*—Continued

CARBOL FUCHSIN

Organism.	Width.	Length.	Average size.
B. atrosepticus	0.4 to 0.7	1.4 to 2.1	0.5 by 1.7
B. solanisaprus	.4 to .6	1.0 to 1.9	.5 by 1.3
B. melanogenes	.4 to .7	1.2 to 2	.5 by 1.6
IIIA	.5 to .8	1.3 to 2.5	.7 by 1.8
SE	.4 to .7	1.3 to 2.1	.5 by 1.6
IIP	.4 to .7	1.3 to 2.1	.5 by 1.7
B. phytophthorus from Appel	.4 to .6	.6 to 1.2	.5 by .9

If the strain known as *B. phytophthorus* from Appel be omitted, it will be seen that, while there are minor variations with different stains, the three organisms from Maine run a little larger than those previously named and described. Of these, IIIA is slightly larger than the other two. Of the remaining three, while *B. solanisaprus* is plainly somewhat smaller, on the average this difference is very slight. Taken as a whole it seems difficult to separate the six different strains on the basis of the morphological characters. It is true that the measurements given differ somewhat from those previously recorded for the described organisms. The writer is not in position to say which are the more accurate, but this does not appear to him to be the important point under consideration. What is given here is the relative measurements from stained preparations of the different organisms, made under as nearly uniform conditions as possible, and measured by the same individual, using the same apparatus for all.

CULTURAL FEATURES

AGAR STROKES.—On agar slants the growth characters of *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, and IIP were identical in every respect except as noted below. The growth was moderate, filiform, with a slight tendency to produce pseudopod-like branches on the surface, if the latter was slightly moist, or such branches might grow up from the bottom of the slant from the condensation water. Elevation flat to slightly raised, luster glistening, topography smooth. Slightly but distinctly bluish opalescent to transmitted light (a smear on the surface of slants with a loop would produce a decidedly opalescent growth). Color pearly white, odor absent, consistency butyrous, slightly viscid in the case of *B. solanisaprus* and *B. melanogenes*. No discoloration of the medium was observed in any of the cultures of the organisms named above.

B. phytophthorus from Appel differed from the above in that the growth, though moderate, was spreading and very finely plumose, giving a characteristic appearance under a low-powered hand lens. *B. phytophthorus*

from Schuster also showed characters on agar slants which differentiated it from all of the other cultures, including the one just mentioned. The growth while filiform at first was more abundant, and later it spread out in a broad band one-third to one-half the width of the slant. It was thicker, somewhat convex and yellowish, and not opalescent. It very soon imparted a very distinct yellowish-brown color to the substratum.

AGAR STABS.—The first six organisms mentioned in the above section produced the same appearance on agar stabs. The growth was fairly uniform but slightly best at the top, although perhaps not more than would result from the heavier inoculation of the upper layers of the medium. The entire surface was covered in from a few days to about a week, depending upon the amount of free moisture present. The line of puncture was filiform to very slightly papillate.

The two organisms carried under the name "*B. phytophthorus*" grew decidedly best at the top, and the one from Schuster caused a distinct browning of the medium, beginning at the surface and diffusing downward.

COOKED POTATO CYLINDERS.—The character of the growth on the cooked potato has been a prominent one among those given by different authors as differentiating from the others in the group the organisms which they have described, consequently considerable attention was given to this topic.

For the work potatoes were chosen which were either freshly dug or had been in storage only a few weeks. In all cases the tests were made on slanted cylinders of sterilized potato in test tubes containing a small amount of distilled water. The tubes were inoculated as soon as sterility was proven, by making a single stroke with a straight needle along the center of the slant.

Inoculations made to tubes of this kind gave practically identical growth characters for *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, and IIP, which are as follows:

At 20° C. little or no growth apparent in 24 hours; needle growth plainly evident in 48 hours, and if the slant is rather moist, the growth may have spread out by this time to cover a considerable portion of the lower part; in three days, growth moderate to abundant, filiform to spreading, depending upon the amount of water in the substratum; one week, growth abundant and spreading, usually covering the entire surface of the slant. Elevation first convex, then slightly raised to flat; luster glistening; topography smooth to slightly rugose; color yellowish white, resembling pus in appearance, later dirty white; odor not apparent at first, but later slight odor of decayed potatoes; consistency butyrous, with a slight tendency to viscidity in the case of *B. solanisaprus* and *B. melanogenes*. Medium at first slightly grayed, and at the end of a week plainly grayed.

The organism received from Appel as *B. phytophthorus* produced no apparent growth upon potato cylinders, but the liquid below was clouded, and the substratum grayed or slightly browned.

The organism received from Schuster as *B. phytophthorus* upon cooked potato agreed with the detailed description of the group given above, except that in the earlier stages the growth was more abundant, and at all times it had a distinct yellow color, especially on the more moist portions of the slant. This agreed quite closely with the "honey-yellow" color which Appel originally described for *B. phytophthorus*. The medium was considerably grayed and had a brownish tinge.

GELATIN STABS.—The behavior of the various named species with reference to liquefaction in gelatin stab cultures had been used also as a differential character. While all are reported as liquefiers, Harrison (17), who did a large amount of work with *B. solanisaprus*, reports this organism as very slow in this respect, liquefaction not beginning until 35 days, and not complete in 44 days. The others are reported to liquefy gelatin much more rapidly, although van Hall (15) stated that with *B. atrosepticus* liquefaction was variable.

Therefore in the present studies particular attention was paid to the behavior of the various organisms in gelatin stabs. With regard to *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, and IIP, the behavior was somewhat erratic on this medium, especially when the "gold label" gelatin was used; sometimes one would liquefy the medium more slowly, and sometimes another. For example, in one case with *B. solanisaprus* liquefaction began on the third day, and was not complete in 60 days. In another series the same organism, incubated at the same temperature, 20° C., showed the beginning of liquefaction in less than 24 hours, which was complete in 7 days, or the shortest time observed for any of the series. After Nelson's photographic gelatin, No. 1, was adopted, the results were much more uniform. So close were these at times that in a series of duplicate or triplicate cultures of the 6 organisms named it would be impossible to pick out those which were of the same name, except for the labels on the tubes.

Therefore the final conclusion was that *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, and the three organisms from Maine present no well-defined differential characters upon gelatin stab which would separate them from each other. The observed characters of the six on gelatin may be briefly summarized as follows: A needle growth was apparent in 12 hours at 20° to 22° C. Liquefaction as a rule begins in from 18 to 24 hours, and may not begin until the third day. It is usually complete in about 10 days, but may be delayed for some time longer. Usually liquefaction is infundibuliform to slightly napiform and later may assume a saccate appearance. Occasionally liquefaction starts off rapidly at the surface, giving a distinct crateriform appearance, which is quite likely to

change to stratiform, and progresses very slowly from that time on. These variations were common to all of the pathogens and were not confined to any one of the series. No change of color or fluorescence was observed. The liquefied medium is quite turbid at first, and a copious flocculent or granular, whitish deposit settles out into the bottom of the funnel.

Of the two cultures received as *B. phytophthorus*, that from Appel did not liquefy gelatin and that from Schuster produced first a crateriform and then a stratiform liquefaction of only the upper portion of the medium, which was browned.

NUTRIENT BROTH.—In neutral nutrient broth (10 c. c. inoculated with a 2-mm. loop of young broth culture) the clouding in the case of *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, and IIP is very rapid. It is apparent in 13 to 16 hours at 20° C., varying somewhat with different strains, very evident in 18 hours, and is quite marked in from 24 to 48 hours. Later moderate to strong, persistent, the fluid being frequently turbid in old cultures. Usually a very slight ring may be observed in young cultures, and occasionally a very slight, granular pellicle is formed during the first few days of growth which readily breaks up and falls to the bottom if the tube is handled or even slightly jarred. The sediment is compact, scant, granular, and dirty white. No odor was observed and no discoloration of the medium. As a rule, a very slight viscidity in both sediment and liquid was noted in the case of *B. solanisaprus* and *B. melanogenes*. Otherwise the six above-named organisms were exactly identical in respect to observed characters upon bouillon.

The organism received from Appel as *B. phytophthorus* differed from the above in that with it the sediment is very adhesive. While it was not strictly viscid it resisted pulling apart with the needle and then appeared very stringy. There was no evidence of viscidity of the medium itself in cultures of this organism. The organism received from Schuster as *B. phytophthorus* differed from all the others in that in old cultures the medium was distinctly browned. While the sediment was dirty white it was rather more abundant, and appeared more stringy and viscid than was the case of the other culture carried under this name.

GROWTH IN POTATO BROTH.—The potato broth used was made as follows: Sound, fresh, recently harvested potato tubers were used and after peeling and washing, 500 gm. were grated directly into 1000 c. c. of distilled water. This potato pulp and water was heated for one hour at a temperature of 55° C. and filtered first through cotton and then through filter paper to remove the starch and pulp. The filtrate was then placed in the steamer for 40 minutes at 99° to 100° C., and again filtered through paper. Titration showed the natural acidity to be equivalent to +10 Fuller's scale and this was not changed. The medium was then tubed and sterilized by fractional steaming—15 minutes at 99° to 100° C.

on each of three consecutive days. It gave a faint blue color on the addition of iodin solution, showing the presence of a small amount of starch.

In ordinary culture tubes the appearance produced by *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, and IIP was absolutely identical, except that the last named produced somewhat more turbidity with less cloudiness. The characters shown were essentially those recorded for beef bouillon except that the growth was somewhat more vigorous and clouding appeared more rapidly, with a greater tendency to produce a ring or pellicle on the surface. This latter was of a granular nature and was less easily broken up than was the case with the pellicle formed on beef bouillon.

In fermentation tubes filled with potato broth there appeared a thorough, uniform clouding of both bulb and closed arm in from 18 to 24 hours, but no gas of any kind was formed.

With the cultures received as *B. phytophthorus* from Appel and as *B. phytophthorus* from Schuster the growth in ordinary culture tubes was the same as in beef bouillon except that with them it was also somewhat more vigorous. In fermentation tubes containing the potato broth *B. phytophthorus* from Appel showed a marked contrast in the appearance of the same organism in fermentation tubes of ordinary beef bouillon containing carbohydrates. With the latter it was never observed to produce any visible clouding of the closed arm of the tube, while in the tubes of potato broth there was a faint clouding of the closed arm with a very perceptible growth on the third day. In the case of *B. phytophthorus* from Schuster the characters were not essentially different from those observed with ordinary bouillon plus carbohydrates. There was a rapid and heavy clouding of the bulb, followed by a slow clouding of the closed arm.

After the cultures had grown for 10 days, the broth was tested with iodin solution, but in no case did there seem to be any diminution of the amount of starch present.

MILK.—Very little difference could be observed in the action of *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, and IIP upon fresh skimmed milk sterilized by fractional steaming, except that SE produced a slow but evident digestion of the curd. With this exception noted the record for the group when grown at a temperature of 20° C. is: Coagulation somewhat delayed, not appearing for about 7 days; extrusion of whey does not take place until the end of 10 to 14 days, and only a slight amount is formed. Coagulum solid and apparently not digested, but readily breaks apart when probed with the needle, not slimy nor viscid, and when shaken forms nearly a solid mass with the whey. It will be noted that both Harrison (17) and Pethybridge and Murphy (26) report coagulation in 48 hours, but this is at 25° C. The latter gentlemen, however, recorded coagulation not appearing for five days at laboratory temperatures.

The two organisms carried under the name of *B. phytophthorus* were not tested upon milk and litmus milk.

LITMUS MILK.—On litmus milk the six organisms mentioned above produced at 20° C. a rather slow, moderate acid development at first, but later the cultures became strongly acid. At this temperature reduction or bleaching begins to appear at the end of one month's time. If the tubes were then heated sufficiently to kill the organisms, the red color promptly returned.

GELATIN COLONIES.—Colonies upon gelatin plate cultures made from meat-extract broth plus 10 per cent by weight of Nelson's photographic gelatin No. 1 and made neutral to phenolphthalein, incubated at 20° C. were identical in the case of *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, and the three Maine organisms. These characters were: Growth rapid, buried colonies in 24 hours, round, margins entire. Under a 16-mm. objective and 12 eye-piece, the colonies at this time appeared nearly white and finely granular, usually with a faint indication of liquefaction at the margin. In 48 hours, in every case, a well-defined crateriform liquefaction had appeared and heavily seeded plates would be largely liquefied by this time at 20° C.

AGAR COLONIES.—The organisms mentioned in the preceding paragraph likewise gave characters identical with each other upon neutral meat extract agar. In 24 hours, viewed under a 16-mm. objective and 6 ocular, the colonies were brownish-yellowish in color, finely granular, margins entire, lens shaped to slightly ovoid or spherical. Viewed under a hand lens the colonies were slightly yellowish in color. The surface colonies were pearly white, bluish opalescent to transmitted light, flat, circular, and occasionally amœboid in shape if the plate were thinly seeded and the surface moist.

Some preliminary work was done, but no detailed studies were made with regard to the colonies produced upon gelatin and agar with the two organisms received and studied as *B. phytophthorus*.

FERMI'S SOLUTION.—In liquid cultures of Fermi's solution all the organisms showed a slight clouding in 24 hours. In 48 hours this was very well defined, but moderate, in the case of *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, and IIP, with a tendency to form a pellicle, which in the case of SE was frequently quite well defined. *B. phytophthorus* from Appel and *B. phytophthorus* from Schuster at the same time had produced only a faint trace of cloudiness in the medium which later disappeared. Later observations showed considerable increase of cloudiness, almost to opaqueness in the case of the first six organisms named.

FERMI'S SOLUTION PLUS AGAR.—No attempt was made to grow the various organisms on Fermi's solution in silicate jelly, but one trial was made in which one and one-half per cent of agar was added and the sterilized tubes of media, slanted as in ordinary beef-peptone agar. On

these agar slants in 24 hours after being inoculated with a single stroke of a straight needle there was no visible growth. In 48 hours and later it was well defined and of uniform appearance in the case of *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, and IIP. The character of the growth may be described as moderate, filiform, flat to slightly raised, smooth, glistening, plainly opalsecent, later opaque, color whitish or pearly, later creamy, odor absent, consistency butyrous—slightly viscid in the case of *B. solanisaprus* and *B. melanogenes*.

The two strains carried as *B. phytophthorus* produced only a very faint, semitransparent, filiform growth on this medium. No discoloration of the medium was observed in either case.

COHN'S SOLUTION.—No growth was obtained with any of the organisms when inoculated into Cohn's solution.

USCHINSKY'S SOLUTION.—The organisms received from Dr. Schuster as *B. phytophthorus* gave little evidence of growth in this medium, while all of the others produced a copious growth and behaved alike in it. Visible clouding with the latter was less during the first 24 hours than in broth tubes inoculated at the same time, but at the end of three days the medium was quite heavily clouded and milky in appearance.

SODIUM CHLORID IN BOUILLON.—One, two, three, four, and five gm. of sodium chlorid to each 100 c. c. of culture media were added to neutral beef infusion bouillon and narrow tubes each containing 5 c. c. of this salted medium were inoculated with a 2-mm. loop in the usual way.

At the end of 18 hours there appeared to be no inhibition of growth of any of the cultures in the tubes containing 1 and 2 per cent of sodium chlorid. In 3 per cent, all were slightly but plainly less clouded than the check tubes inoculated at the same time, and no growth had appeared in the 4 and 5 per cent. At the end of 48 hours all the tubes containing 3 per cent of sodium chlorid were apparently as well clouded as the checks. All showed growth in the presence of 4 per cent, but with some inhibition. In 5 per cent no growth was apparent in any of the tubes except those of *B. phytophthorus* from Appel, which showed about half-normal clouding for bouillon cultures of the same age. In three days *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, and IIP in 5 per cent of sodium chlorid showed a faint but well-defined clouding for the first time and appeared quite uniform in appearance. *B. phytophthorus* from Appel was quite heavily clouded, while no growth could be detected in the culture of *B. phytophthorus* from Schuster.

GROWTH IN BOUILLON OVER CHLOROFORM.—This test was made with tubes containing 10 c. c. of sterile bouillon into each of which were introduced 5 c. c. of chloroform and the whole thoroughly agitated at intervals for two days. After allowing the tubes to incubate for several days to prove sterility they were inoculated with a 2-mm. loop of broth culture in the usual way, care being taken this time to agitate the medium

as little as possible and to introduce the inoculating loop only into the upper layers of the culture liquid.

In 18 hours there was no growth in any except *B. phytophthorus* from Schuster which showed a little clouding in the upper layers. Daily observations were made following this for several days.

In 36 to 48 hours as compared with normal broth cultures inoculated at the same time those of *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, and IIP were much restrained, but there was an evident slight clouding all through, slightly stronger at the top. The same condition prevailed as long as the cultures were under observation, except that after three or four days the three organisms from Maine showed a more vigorous growth than the other three and produced a distinct, moderate clouding all through the bouillon. The appearance of the first three named was identical in all respects.

The growth in the cultures known as *B. phytophthorus* from Appel and from Schuster after about 48 hours was unrestrained and appeared equally as abundant as that in the check tubes.

BEST MEDIA FOR LONG-CONTINUED GROWTH.—Of the various media used, neutral beef bouillon, made either from meat infusion or from Liebig's extract, proved to be the best for long-continued growth.

PHYSICAL AND BIOCHEMICAL FEATURES

FERMENTATION OF CARBOHYDRATES.—The ability of the different organisms in this group to ferment various carbohydrates has furnished perhaps the most important differential characters upon which the previously named species have been erected. Therefore in the present studies more attention has been given to this subject than to any other. The results obtained are not based upon single trials, but upon repeated tests of each organism. All the fermentation tests were made in uniform fermentation tubes, having a small neck, a large bulb, and a capacity of about 25 c. c. in the closed arm. These were made to order and very closely conform to the illustration given by Smith (32, v. 1, p. 53). Both meat-infusion bouillon, previously freed from muscle sugar by inoculation with *B. coli*, and meat-extract bouillon made from Liebig's extract and tested for the absence of fermentable carbohydrates, as previously described, were used. The meat-extract bouillon was used for a large proportion of the work, for it was found to be more satisfactory. For the fermentation work 1 per cent of the carbohydrate used was added to the culture medium and the tubes containing the media were sterilized by fractional steaming. The following substances were tested for fermentation in this way: Dextrose, saccharose, lactose, maltose, glycerin, mannit, and dextrin. In addition some of the organisms were tested in the neutral-red lactose broth which is commonly used in water work as one of the presumptive tests for *B. coli*. The culture medium in each case was made neutral to phenolphthalein.

To save unnecessary repetition it may be stated in the beginning that the two organisms received and carried under the name of *B. phytophthorus* produced no gas or acid in the presence of any of these substances and imparted a slight alkalinity to the culture medium. The one received from Dr. Appel in no case produced any visible clouding of the closed arm of the fermentation tube. The one received from Dr. Schuster produced a delayed clouding of the closed arm in all cases except with glycerin, but even with this the clouding was later nearly as complete as in the bulb.

The results obtained with the remaining organisms—namely, *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, IIP—were alike and constant with regard to gas formation, growth in the closed arm, and production of acid. On repeated trials they all invariably produced gas in the presence of dextrose, saccharose, lactose, maltose, and mannit. They produced no gas from glycerin and dextrin. With dextrose this amount of gas was small, ranging from 3 to 5 per cent. With saccharose the percentage of gas was higher, usually being from 7 to 9 per cent. Except with glycerin, there was always a prompt clouding of the closed arm, though this was usually less than in the bulb. This clouding in the closed arm was persistent, although a slight clearing at the top of the tube was observed in old cultures containing maltose. Acid was produced in the presence of all the carbohydrates used.

Very little of the gas which was formed was absorbed by a 2 per cent solution of sodium hydrate. The remainder of the gas was explosive. When expressed in the terms of $H:CO_2$, as accurately as could be determined, this varied from 1:0 to 5 or 6:1. No attempt was made to determine further the nature of this gas. Gas in all cases usually appeared on the second or third day and reached its maximum before the tenth, usually on the sixth or seventh.

It will be noted that this constant appearance of gas in dextrose, saccharose, and maltose with *B. solanisaprus* was contrary to Harrison's original description (17). Likewise the production of gas with *B. atrosepticus* from lactose and dextrose differs from the results recorded by Van Hall. In gas production *B. melanogenes* agreed in every respect to Pethybridge and Murphy's description (26).

In neutral-red lactose fermentation broth the amount of gas obtained in each case with the 6 different organisms was the same as with the ordinary lactose broth. In 48 hours the entire contents of the tube appeared distinctly more red than the check. On the third day the closed arm took on a yellowish olive tinge, which later changed to a canary-yellow, stronger even than that produced by *B. coli*, run at the same time for comparison. This color persisted for one month, or as long as the cultures were under observation. During the same time the bulb showed a more pronounced red than the check tube.

AMMONIA PRODUCTION.—Cultures in Dunham's peptone solution were tested at the end of one, two, three, and four days as follows: In clean test tubes of the same internal diameter there were placed 10 c. c. of ammonia-free water. Into each was placed one 2-mm. loopful of the culture to be tested, taken from the top of the tube, and then six drops of Nessler's solution was added to each tube of water and culture dilution. The color of the tubes was observed by looking vertically through them upon a white background. No color developed in any of the tubes except in those containing some of the culture of *B. phytophthorus* from Schuster. The latter gave a distinct yellow color.

Pethybridge and Murphy (26) have recorded the appearance of small amounts of gas in cultures of *B. melanogenes* in tubes of a 2 per cent potassium-nitrate broth having a plug of vaseline on the top. For the purpose of testing all of the organisms in this respect, fermentation tubes were filled with the nitrate broth described in the next section, sterilized by fractional steaming, and then inoculated. Growth appeared in these rather slowly but at the end of 48 hours all were uniformly though somewhat faintly clouded with the exception of the two strains carried under the name of *B. phytophthorus*. These showed only growth in the bulb. Later they showed some clouding of the closed arm of the tube, but this was exceedingly faint in the case of *B. phytophthorus* from Appel. No gas whatever appeared in the closed arm of the tubes with any of the organisms studied. Tubes of this same broth, tested at the end of five days, with Nessler's reagent by dropping five or six drops of the reagent directly into the tubes gave no qualitative test for ammonia, except in the case of *B. phytophthorus* from Schuster. Cultures of the latter when treated with the reagent produced a very distinct, yellow reaction. That the Nessler's solution used was of good quality was indicated by the fact that it would produce the characteristic reaction in the presence of very minute quantities of ammonia, artificially introduced into distilled water.

In connection with these tests it should be noted that Pethybridge and Murphy used a 2 per cent potassium-nitrate broth, while the writer used that specified by the Committee on Standard Methods of Water Analysis,[1] which carries only 2 gm. of potassium nitrate per liter.

Where Nessler's solution was added directly to young cultures in Dunham's peptone solution, in no case was there any more color observed to result than where the uninoculated check tube of the same medium was so treated, except in the case of *B. phytophthorus* from Schuster. Cultures of this organism gave a yellow color reaction.

REDUCTION OF NITRATES.—Cultures in nitrate broth, consisting of 2 gm. of chemically pure potassium nitrate, 1 gm. of Witte's peptone and 1 liter of water, were tested by the starch-iodin method after five days. All

[1] Report of committee on standard methods of water analysis to the laboratory section of the American Public Health Association, presented at the Havana meeting, January 9, 1905. 141 p. Chicago, Ill., 1905. Reprinted from Jour. Infect. Diseases, suppl. 1, 1905.

except *B. phytophthorus* from Appel when so tested at once showed a deep-blue color reaction, indicating the presence of nitrites. No such color appeared in the uninoculated check tubes of the same medium tested at the same time.

From what is said in the preceding section it might be inferred that *B. phytophthorus* from Schuster is apparently able to reduce nitrates to nitrites and then to ammonia. However, it should be remembered that the nitrate broth used contained a small quantity of peptone, and it has been shown that the organism is able to produce ammonia from peptone. Doubtless this was the source of the ammonia reaction in the nitrate broth.

INDOL PRODUCTION.—Cultures of *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, the three Maine organisms and of the one received from Schuster as *B. phytophthorus* gave a positive reaction to the indol test. That received from Appel as *B. phytophthorus* and the check tubes always gave a negative reaction. The pink color, while well defined, and sometimes in old cultures being quite marked, was very much less than that produced by *B. coli*, which was tested at the same time for comparison. In the case of the positive-reacting organisms the color was very slight or absent in cultures four to seven days old and was most marked in cultures two months old or more. Therefore, indol production where it occurred would be classed as being feeble to moderate.

TOLERATION OF ACIDS.—In the present studies only hydrochloric acid has been tested. Normal hydrochloric acid was added to meat-extract broth which in the beginning was neutral to phenolphthalein, in sufficient quantities to make it +10, +20, +30, +40, and +50 Fuller's scale.

In from 12 to 18 hours all of the organisms showed equally good growth in the check tubes of neutral broth. At +10 the amount of clouding was nearly equal to that produced in the checks. At this time there was a marked falling off in the clouding at +20 as compared with the check tubes, and no growth had appeared in the presence of the larger amounts of acid.

At 48 hours and later there was no change in the relative amounts of cloudiness exhibited by the different organisms, except that cultures of *B. phytophthorus* from Appel were slightly less cloudy and *B. phytophthorus* from Schuster were slightly more cloudy at +20 than those of *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, and IIP. The latter were all alike in their behavior toward hydrochloric acid. None of the various organisms produced any growth at +30 or beyond.

TOLERATION OF SODIUM HYDRATES.—Cultures of all of the organisms were made in meat-extract bouillon, neutral to phenolphthalein, in comparison with the same medium made −10, −20, −30, −40, and −50 Fuller's scale with normal sodium-hydrate solution.

At the end of 12 to 18 hours the cultures in the −10 bouillon were as heavily clouded as the neutral; those at −20 were well clouded but plainly less than at −10. At this time *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, IIIA, SE, and IIP were practically identical in the amount of cloudiness they produced on the different media, but showed no growth beyond −20. *B. phytophthorus* from Appel and *B. phytophthorus* from Schuster showed a decreasing amount of cloudiness up to and including −40, but they showed no growth in the tubes of media with a reaction of −50.

In 48 hours the last two named had produced a faint but evident growth in the medium having a reaction of −30. At the end of two weeks all showed growth at −40 and all but *B. atrosepticus* and SE at −50.

OPTIMUM REACTION FOR GROWTH IN BOUILLON.—This is approximately the same for all of the organisms studied. It lies very close to the neutral point of phenolphthalein, but is apparently on the alkaline side. Observations to determine this must be made with these organisms during the first 12 to 18 hours of growth. Broth cultures with a reaction of +10 with hydrochloric acid and −10 with sodium hydrate, Fuller's scale, were practically equally well clouded in 24 hours, but the acid cultures in the early stages of growth were observed to cloud slightly more slowly. Sodium hydrate restrains the growth much less than does hydrochloric acid, but in both alkaline and acid media of stronger reaction than that first mentioned there was a very decided falling off in the clouding during the first 24 hours as compared with that obtained with media of neutral reaction.

VITALITY ON CULTURE MEDIA.—Cultures of *B. solanisaprus* and the three organisms from Maine in neutral beef-extract bouillon, stored at a temperature of from 15° to 18° C., were found to be alive at the end of 8 to 10 months, provided the moisture had not entirely evaporated. Tests were made with the other organisms, but with somewhat younger cultures and in each case they were found to be alive in the bouillon. Cultures in liquefied gelatin were also found to possess long vitality, but upon agar slants, milk, and the various carbohydrate broths, the organisms were killed out much more readily. Those in milk were frequently found to be entirely dead at the end of three months.

TEMPERATURE RELATIONS.—The optimum temperature for growth in the six pathogens studied is not far from 25° C., although no attempt was made to determine this within a variation of 5° C. above and below. Numerous tests were made of all the strains at the same time in seven different incubator chambers, running from 5° to 35° C. Considerable differences were noted in the rates of growth at lower temperatures, even in different tests of the same strain, but as the optimum was approached a striking uniformity was obtained. This is well illustrated by Table III, which gives the results secured from a single series. In the temperature

columns of this table are given the number of hours which elapsed from the time a 10-c. c. broth culture was inoculated with a 2-mm. loop of a 24-hour-old broth culture, before the first visible traces of clouding of the medium could be observed, observations being made hourly. In all cases the tubes of media were placed in the incubator at the desired temperatures for a few hours before inoculation, and care was taken not to remove them therefrom any longer time than was necessary to make the inoculations and observations.

TABLE III.—*Effect of temperature on rate of growth of the organisms*

Organism.	First evidence of clouding of broth cultures at—						
	5° C.	10° C.	15° C	20° C	25° C.	30° C.	35° C.
	Hours.	*Hours.*	*Hours.*	*Hours.*	*Hours.*	*Hours.*	*Hours.*
B. atrosepticus	84	27	22	13	11	12	No growth.
B. solanisaprus	96	27	22	13	11	12	Do.
B. melanogenes	52	27	22	13	11	12	Do.
IIIA	84	28	21	14	11	12	Do.
SE	96	27	21	16	11	12	Do.
IIP	192	59	27	16	13	16	Do.
B. phytophthorus from Appel	192	64	30	18	13	12	16.
B. phytophthorus from Schluster.	192	60	22	13	8	11	12.

It will be seen that while the optimum for the two nonpathogenic strains carried under the name of *B. phytophthorus* is about the same temperature as for the six pathogens, the former grew at 35° C., while the latter did not.

Both agar slants and beef-broth cultures were used to determine the maximum temperature for growth in the case of *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes*, and the three pathogenic strains isolated in Maine. On beef-extract broth neutral to phenolphthalein this was not far from 33° C.; if anything, slightly below this. None of the six produced visible clouding of broth at 34° C. and above. On agar slants the growth was scanty or absent at 32° and 32.5°, *B. atrosepticus*, SE, and IIP giving the most frequent failures to produce growth on agar at these temperatures. All of the pathogenic strains would show very evident growth in broth at 32° in 18 hours, and also clouding in the same period of time, with less regularity and with some failures to produce clouding at all at 32.5°. Undoubtedly the somewhat lower maximum temperature for growth on agar was due to the rapid drying out of the surface of the medium. The results given above were checked a number of times, since they were unexpected on account of Harrison having reported growth with *B. solanisaprus* at 37°.

The thermal death point for *B. atrosepticus*, *B. solanisaprus*, *B. melanogenes* and the three pathogenic strains isolated from Maine was found to be approximately 46.5° C. Retests at this temperature did not

always prevent later clouding of the inoculated and heated tubes of broth. In no case, except with a single tube of *B. melanogenes*, did growth occur after heating at 47° and above, but clouding always appeared after an exposure to 46° C. and below. Transfers from well-clouded broth cultures 36 to 48 hours old appeared more resistant than those a few days older. The strains carried under the name of *B. phytophthorus* showed a much higher thermal death point. For the culture received from Dr. Appel it was between 51° and 52°, while that from Dr. Schuster was killed at 54° but not at 53°.

In making the thermal-death-point determinations thin-walled test tubes of 16 to 17 mm. internal diameter and approximately 16 cm. long were employed. Each tube contained 10 c. c. of meat-extract broth, +15 Fuller's scale.

Immediately after inoculation they were transferred to a specially constructed water bath, provided with a stirring apparatus and an accurate, certified thermometer, and immersed nearly their entire length in the heated, constantly moving water. The period of immersion was 10 minutes and the bath during this time was maintained within one-tenth of 1 degree C. of the required temperature.

EFFECTS OF DRYING.—The effect of drying was tested as follows: A 2-mm. loop of a 24-hour broth culture was removed to and spread upon small, sterilized, cover glasses in sterile petri dishes. These were allowed to dry at the same temperature as incubation for varying periods after the last trace of moisture had disappeared from the cover glasses. Then the latter were picked up with flamed forceps and dropped into tubes of sterile bouillon. Only *B. solanisaprus*, IIIA, and SE were tested in this way. The results were somewhat variable. Usually one or two minutes' drying were sufficient to produce sterility, although in one or two cases growth appeared after 10 and 15 minutes' drying. However, in each case like this the growth was much retarded.

EFFECT OF SUNLIGHT.—The effect of sunlight was tested only in the case of the three organisms mentioned in the preceding paragraph. The usual method was followed: The freshly inoculated and half-covered petri-dish cultures were exposed on blocks of ice to bright sunlight at midday in a greenhouse. There was no diminution of resulting colonies on the exposed side in 10-and 20-minute exposures, slight diminution at 30 minutes, while at 60 minutes all the organisms on the exposed side were killed, and no colonies developed. This test was made in November and consequently the conditions were not favorable. Doubtless the same exposure outdoors in midsummer would have shown sunlight to be more effective in killing the organisms.

EFFECT OF GERMICIDES.—In testing the effect of germicides, only mercuric chlorid and formaldehyde were employed, and only *B. solanisaprus*, IIIA, and SE were tested. The results were the same with

all three organisms. In the case of the mercuric-chlorid test one 2-mm. loop of a deeply clouded, 24-hour broth culture was transferred to check tubes of sterile distilled water and to others containing varying dilutions of the poison, the weakest being 1 to 50,000. After one hour transfers were made from these to fresh broth tubes. In no case was growth obtained except from the inoculated check tubes of pure water. Where mercuric chlorid was added directly to broth tubes it was found that it required a concentration of 1 to 10,000 to produce the same results.

With formaldehyde no attempt was made to determine how weak a solution would be effective. It was simply tested and found that one part in 250 in distilled water or beef broth was sufficient to kill all organisms in an hour's exposure. This was somewhat weaker than is usually used for disinfecting potato tubers and the time one-half as long.

PATHOGENICITY

As has already been stated, all of the strains studied were tested at various times to determine their ability to produce the characteristic blackleg disease of the stem and softrot of the tuber. From the beginning to the close of the work *B. solanisaprus*, *B. melanogenes*, and the three strains from Maine were found to be pathogenic to potato stems, leaf stalks, and tubers.

When first received in March, 1910, the culture of *B.atrosepticus* either failed to attack or caused a slow decay of pieces of potato tubers produced the season before, which had been cut under aseptic conditions and placed in tubes of sterile water. However, after several repeated transfers from one 24-hour-old potato-broth culture to another it was found to produce a fairly rapid decay when inoculated into potato tubers, especially those which were immature and recently dug. This strain has continued to rot tubers, provided they were not too mature and care was taken not to allow the inoculated portion to dry out too rapidly..

It has been the custom whenever the pathogens were tested on growing stems of potato plants to inoculate a plant each time with each of the nonpathogenic strains. *B. atrosepticus* was repeatedly used in this way, but the results were negative previous to June 15, 1916. A plant inoculated on this date by puncturing the young, growing stem near the base with the needle of a hypodermic syringe and inserting a small amount of a 24-hour-old culture in the tissues of the pith, was observed five days later to be showing slight blackening around the point of inoculation. Closer examination showed that the pith had been softened and decayed for a short distance above the puncture. This diseased condition did not progress much farther, however. About a month later several other young stems were inoculated with transfers of the stock strain of *B. atrosepticus* and this time the positive results were more marked. The inoculated stems were blackened on the surface for a dis-

tance of 3 to 4 inches and finally fully decayed and cut off, resulting in the death of that part of the plants above the points of inoculation.[1]

As has been clearly indicated in the previous pages, inoculations of potato stems and tubers with the cultures received and carried under the name of *B. phytophthorus* produced no disease whatever. The punctures into which the cultures were inserted dried out rapidly and were in every way similar to those into which sterile broth or distilled water had been inserted as checks.

Some work was done to test the pathogenicity of the strains used, upon plants closely related to and unrelated to the potato. It will be unnecessary to go into the details of this since the present studies are primarily concerned with a comparison of the various morphological, cultural, physical, and biochemical features of the different organisms.

GROUP NUMBERS

The numerical system employed in the descriptive chart or card adopted by the Society of American Bacteriologists furnishes a means whereby a considerable number of contrasted, salient characters may be brought together in a small space to facilitate comparisons. This decimal system of group numbers is useful as a quick method of showing close relationships and important differences within the genus, but is not a sufficient characterization for any organism. However, it does record in a very compact manner many of the important differential characters.

GROUP NUMBERS BASED ON THE PRESENT STUDIES

B. atrosepticus Van Hall	221. 1113522
B. solanisaprus Harrison	221. 1113522
B. melanogenes Pethybridge and Murphy	221. 1113522
IIIA	221. 1113522
SE	221. 1113522
IIP	221. 1113522
B. phytophthorus (as received from Appel)	222. 3333033
B. phytophthorus (as received from Schuster)	221. 3333533

Some question might be raised with regard to whether the figure in the fifth place to the right of the decimal point in the first six group numbers should be a 5 or a 0. Here the only yellow color that developed was on potato, and this was by no means strong. It is possible also that the next figure to the right in the same group numbers should be 3, for the diastatic action on potato starch is doubtful.

[1] The writer has no explanation to offer for this unexpected manifestation of pathogenicity on the part of this culture of *B. atrosepticus*. The system used in keeping and transferring the stock cultures is such that any possibility of getting the different strains mixed is practically eliminated. The fact that the stock cultures have always been carried in beef broth with occasional transfers to potato broth may have something to do with it. Others who have kept their stock cultures of these and the closely related softrot bacteria of the *B. carotovorus* type on agar have reported loss in pathogenicity. Including those mentioned in this paper the writer has carried over 20 different strains of blackleg organisms for from 5 to 9 years, but with no permanent evidence of loss of pathogenicity with any.

In the case of the organism received from Schuster and carried under the name of *B. phytophthorus*, the yellow growth was apparent on both agar and potato. Upon the latter the growth was of a very distinct lemon yellow, considerably deeper than that produced by the other organisms.

NOMENCLATURE AND RELATIONSHIP

An analysis of the data obtained as the result of these comparative studies seems to point to but one conclusion. When subjected to the various differential tests at the same time and under the same conditions, the cultures received under the names "*Bacillus atrosepticus* Van Hall," "*Bacillus solanisaprus* Harrison," and "*Bacillus melanogenes* Pethybridge and Murphy" and the three strains of organisms isolated from potato plants affected with the blackleg disease from widely separated parts of Maine appear to be identical.

It is the writer's opinion that the pathogenic organisms studied should be classed as one species, or at the most strains of the same species. The only constant differences noted were slight variations in size, shown more particularly by *B. solanisaprus* and IIIA, and the production of a slight viscidity on different kinds of media shown by *B. solanisaprus* and *B. melanogenes*, but not by any of the others.

It is evident that the two organisms which the writer received and studied under the name of *Bacillus phytophthorus* Appel were not alike, nor were they the same as the one originally described by Dr. Appel.

There is nothing in the data here presented which bears on the relationship between the organism originally described by Dr. Appel as *B. phytophthorus* and the other strains of blackleg bacteria. As has already been stated, Dr. Smith states that it and *B. solanisaprus* are not identical, but are closely related. While the writer regrets that he was unable to get an authentic, virulent culture of this organism for comparison in time to make use of it he has no reason for questioning this statement, coming from so good an authority.

While the present studies are primarily concerned with relationship, the results obtained make it impossible to entirely ignore the question of nomenclature. If it is granted that *B. phytophthorus* differs from *B. solanisaprus* and consequently from the others under consideration, which of the three other names should be retained? On grounds of priority, *B. melanogenes* is excluded and the choice is between *B. atrosepticus* and *B. solanisaprus*. Also on the grounds of priority alone it would seem that the accepted name should be *B. atrosepticus*, but for certain reasons the writer was at first inclined to favor *B. solanisaprus*. These reasons are as follows: According to present standards *B. atrosepticus* was not very fully described in the beginning. Moreover, the culture used by the writer was obtained from Kral's laboratory with no statement regarding its origin or authenticity. Also its pathogenicity was erratic or weak,

and for a long time it entirely failed to produce typical blackleg of the stem upon inoculation. This was in accord with the impression one would gain as to Van Hall's results relative to pathogenicity on reading certain reviews of his paper (15). When Dr. De Zeeuw's translation of Van Hall's paper became available, the results of his inoculation experiments appeared in quite a different light. These were quite limited, on account of a lack of a sufficient amount of proper material. As is the case with any of the pathogenic strains, the inoculation experiments with mature stems and tubers gave erratic results or failures. He was able in some instances to produce typical blackening and decay when young parts of stems were inoculated.

B. solanisaprus, on the other hand, was originally described in much detail, and authentic, virulent cultures are to be obtained by anyone who may wish to study it. That used by the writer is as pathogenic to-day to potato stems and tubers as when described by Harrison 10 years ago.

In recommending the dropping of the name "*B. melanogenes*," the writer does not wish to be understood as casting any reflections upon the work of Pethybridge and Murphy, or upon their conclusions, based upon the results obtained by them when compared with published descriptions. The data obtained in the present studies have checked in every essential detail with their description of this organism except that the writer secured a positive test for indol and was unable to get the formation of gas in nitrate broth. However, attention has already been called to the fact that Pethybridge and Murphy used a broth containing a much greater percentage of potassium nitrate than was used by the writer. It should be noted that the agreement between *B. solanisaprus* and *B. melanogenes* resulted from the writer's obtaining certain results, especially with the fermentation of the carbohydrates and the liquefaction of gelatin, which were different from those reported by Harrison. Also Harrison reports the thermal death point for *B. solanisaprus* to be 54° C., while Pethybridge and Murphy say that for *B. melanogenes* it lies between 45° and 50°. The writer's results showing that the thermal death point is approximately 46.5° are then in accord with the latter statement.

REVISED DESCRIPTION OF BACILLUS ATROSEPTICUS VAN HALL, GROUP NUMBER 221.1113522 [1]

I. MORPHOLOGY

Vegetative cells. Medium used was an agar slope at 20° C. 1 to 2 days old. Form, short rods, long rods, short chains, long chains. Limits of size in microns (stained preparations) 0.4 to 0.8 by 1 to 2 or more. Size of majority 0.5 to 0.6 by 1.5 to 2. Ends rounded.

Endospores, none.

Flagella, few, not over 6 or 8. Attachment, peritrichiate. Stained by modified Pitfield method.

[1] See statement following the list of group numbers on p. 120.

Capsules, none.

Pseudozoogloea, present, slight. Involution forms, not observed. (Reported by Harrison for *B. solanisaprus*, at higher temperatures.)

Staining reactions. Stains well in aqueous gentian violet, anilin water gentian violet, aqueous methylene blue, alkaline methylene blue, aqueous fuchsin, carbol fuchsin. Gram, negative.

II. CULTURAL FEATURES

Agar stroke. Growth, moderate. Form of growth, filiform. Elevation, flat to slightly raised. Luster, glistening. Topography, smooth. Optical characters, slightly but distinctly bluish opalescent. Chromogenesis, pearly white. Odor, absent. Consistency, butyrous—some strains slightly viscid, others not.

Potato. Growth, moderate to abundant. Form of growth, filiform to spreading. Elevation, first convex, then slightly raised to flat. Luster, glistening. Topography, smooth to slightly rugose. Chromogenesis, yellowish white, later dirty white. Slight odor of decayed potatoes in old cultures. Consistency, butyrous—some strains slightly viscid, others not. Medium, slightly grayed at first, later plainly grayed.

Agar stab. Growth, slightly best at top, abundant, widespreading. Line of puncture, filiform to slightly papillate.

Gelatin stab. Growth, best at top. Line of puncture, filiform. Liquefaction, infundibuliform to slightly napiform, later may be saccate, occasionally crateriform to stratiform. Begins in 1 to 3 days at 20° C. Complete in 7 to 10 days with some cultures, in others not complete in 60 days.

Nutrient broth. Surface growth, usually slight ring and occasionally slight granular pellicle in young cultures. Clouding, moderate to strong, persistent. Medium, not discolored. Odor, absent. Sediment, compact, scant, granular, and dirty white—some strains slightly viscid, others not.

Milk. Coagulation, usually not until the seventh day at 20° C. Coagulum, not digested or at the most very slowly peptonized. Medium, not discolored.

Litmus milk. Acid, litmus reduced.

Gelatin colonies. Growth rapid at 20° C. Form, round. Edge, entire. Liquefaction, saucer.

Agar colonies. Growth, rapid at 20° C. Form, round, occasionally irregular, buried colonies, lens-shaped to slightly ovoid or spherical. Surface, smooth. Elevation, flat to slightly raised. Edge, buried colonies, entire, surface colonies, entire to undulate. Internal structure, finely granular. Color buried colonies, brownish yellow under a 16 mm. objective and 6 ocular, slightly yellowish under hand lens, surface colonies, pearly white, bluish opalescent to transmitted light.

Fermi's solution. Growth, first moderate, later abundant.

Cohn's solution. Growth, absent.

Uschinsky's solution. Growth, copious.

Sodium chloride in bouillon. Growth inhibited, 3 per cent, slightly, 4 per cent, some, and 5 per cent, considerably.

Growth in bouillon over chloroform. Growth, much restrained at first, later moderate.

Best medium for long-continued growth. Neutral beef bouillon.

III. PHYSICAL AND BIOCHEMICAL FEATURES

Fermentation tubes. Gas produced from dextrose, saccharose, lactose, maltose, and mannit, but not in glycerin and dextrin. Growth in the closed arm with dextrose, saccharose lactose, maltose, mannit, and dextrin, but either absent or slight with glycerin. Acid produced from dextrose, saccharose, lactose, maltose, mannit, and glycerin.

Ammonia production, absent.

Nitrates in nitrate broth reduced to nitrites.

Indol production, moderate in old cultures, absent or feeble in young cultures.

Toleration of acids. Grows in broth at +20 Fuller's scale and not at +30 with hydrochloric acid.

Toleration of sodium hydrate, great.

Optimum reaction for growth in bouillon in terms of Fuller's scale, 0 or slightly alkaline.

Vitality on culture media. Relatively brief on agar and media containing carbohydrates; long on bouillon.

Temperature relations. Thermal death point, approximately 46.5° C. Optimum temperature for growth, about 25° C. Maximum temperature for growth, about 33° C. Minimum temperature for growth, below 5° C.

Effects of drying. Readily killed.

Exposure to sunlight. Exposure on ice at midday in November, slight diminution at 30 minutes, 100 per cent killed at 60 minutes.

IV. PATHOGENICITY

Virulent strains produce rapid decay accompanied by a pronounced blackening of the surface tissues when inoculated into young, growing potato stems; also causes a rapid softrot of potato tubers at moderate temperatures and may produce decay when inoculated into a considerable number of fleshy vegetables.

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EFFECT OF FERTILIZERS ON THE COMPOSITION AND QUALITY OF ORANGES[1]

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INTRODUCTION

Of the great amount of work which has been done with fertilizers, only a relatively small proportion deals with their effects on the quality of the crop. This is especially true of fruits, partly because of the length of time required and partly because of the conflicting factors which enter into a long-time experiment.

The material presented in this paper has to do with oranges (*Citrus aurantium*). The quality of this crop may offer an easier subject for study than most fruits, as the factors affecting it, such as the percentages of sugar and acid, the texture of the fruit as a whole (its specific gravity), and the proportions of juice and rind, can be accurately measured.

HISTORICAL REVIEW

There have been a number of theories and expressions of opinions put forth as to the effect of the usual fertilizers on the composition of oranges. Webber (7), basing his conclusions largely on the experience of intelligent orange growers in Florida, wrote:

(1) By a proper combination of the various elements used in fertilization one can undoubtedly largely govern the quality and flavor of the fruit.

(2) To obtain a fruit with thin rind, use nitrogen from inorganic sources in moderate quantities, with considerable potash and lime.

(3) To sweeten the fruit, use sulphate of ammonia in considerable abundance, decreasing the amount of potash.

(4) To render the fruit more acid, increase the amount of potash and use nitrogen from organic sources.

(5) If it is desired to increase the size of the fruit, as is sometimes the case, apply a comparatively heavy dressing of nitrogen in some organic form and slightly decrease the other elements. In the case of the tangerine and mandarin, where a larger size is usually desired, a heavy dressing of nitrogen fertilizers would favor this end, and is not objectionable unless carried to excess.

[1] Paper No. 32, Citrus Experiment Station, College of Agriculture, University of California, Riverside, Cal.

These conclusions were accepted by Rolfs (6, p. 19).[1] Hart (4, p. 565) states:

> Closely connected with the subject of soil is that of fertilizers, by the understanding use of which almost any kind of orange may be, as it were, manufactured to order. Free use of potash thickens and toughens the rind, giving the firmness and durability requisite to bear transportation and rough handling, but at the expense of saccharine qualities. The latter may be increased and acidulous qualities modified by the use of more phosphoric acid. Highly nitrogenous applications give fruit surcharged with insipid juice, and cause a lush growth of wood that never properly ripens, inviting attacks of insects and fungi. Composts from the barnyard, or those containing much decomposing animal matter, must be used sparingly, if at all. The most wholesome growth and finest-flavored fruit comes from judicious employment of the best high-grade commercial fertilizers.

The first conclusions based on experimental work in Florida are apparently those of Collison (2). In the valuable report presented he has tabulated a large number of analyses of oranges from trees receiving high- and low-potash fertilizers and high- and low-phosphoric-acid fertilizers. He finds that a high-potash fertilizer does not make a sweeter fruit than the low-potash, "contrary to the very general opinion," but that much potash does increase the amount of acid, an opinion previously reported by Webber. Collison found further that phosphoric acid "probably had no material influence on the variation in the sugar and acid content of the fruit." Unfortunately, he was unable to obtain satisfactory samples from which to draw conclusions in regard to the effect of nitrogen.

The figures on which this paper was based have since been published in full (3).

Colby (1) has made a large number of analyses of oranges grown commercially. In discussing the results obtained, Hilgard (5, p. 181), after discussing the unsatisfactory nature of the experiment (due to poor sampling, conflicting factors, and the short period of time), concluded as follows:

> The oranges not fertilized (No. 2), those fertilized with potash and nitrogen (No. 8), and with nitrate of soda (No. 3) are all about alike, though No. 8 was a little sweeter than No. 2. The latter is a pleasant orange, though somewhat lacking in character (i. e., sprightliness and tartness).
>
> In character the oranges from the "Complete fertilizer" (No. 10) are above those fertilized with nitrate of soda alone (No. 4), and while high in flavor, are not equal to those from the superphosphate group (No. 4).
>
> The highest citrus qualities are possessed by the oranges fertilized with superphosphate alone (No. 4), and next by those from the "Complete fertilizer" (No. 10), and both rank high in flavor.
>
> In sweetness the oranges fertilized with potash and nitrogen (No. 8) and with nitrate of soda are above those not fertilized.
>
> The oranges from the nitrogen and phosphoric acid lot (No. 6) are not as good as those from the superphosphate alone (No. 4), and have less character than those not fertilized (No. 2). Those from the potash and phosphoric acid lot (No. 9) are not of as good quality and are more variable than the above.

[1] Reference is made by number to "Literature cited," p. 138.

From the ultimate analyses of the oranges, Colby found the lowest nitrogen content in those samples which had received no nitrogenous fertilizer, from which he deduced a lack of nitrogen in the soil. The samples not receiving phosphates showed the lowest percentage of phosphoric acid, from which the need of a fertilizer of that nature was deduced. The percentage of potash was not appreciably affected by the presence or absence of fertilizers, showing that potash was not deficient in the soil.

DESCRIPTION OF THE EXPERIMENTAL PLOTS

The University of California Citrus Experiment Station has maintained a continuous fertilizer experiment at Riverside with Citrus trees since 1907. The experiment is composed of 20 plots, with six trees each of Washington Navel oranges, Valencia oranges, Eureka lemons, and Lisbon lemons in each plot. The experiment as laid out consists of four tiers of plots from east to west and five from north to south. The ground slopes from northwest to southeast. The soil is not entirely uniform, being a fairly light sandy loam at the upper end and considerably heavier, with a denser subsoil, at the lower end. Pipe lines are run at the head of each tier of plots in order to provide for the independent irrigation of each plot. The trees of each plot have been managed as uniformly as possible.

The plan of the experiment provided for the addition of the same quantities of the different elements of fertility, from whatever source derived. Every plot receiving a nitrogenous fertilizer, for example, has received the same quantity of nitrogen. Up to the end of 1914, the total amounts applied to each tree had been approximately 6 pounds of nitrogen and potash each, and 12 pounds of phosphoric acid.

EXPERIMENTAL WORK

A study of the quality and composition of the fruit was first taken up with the crop of 1914. In this work a sample of 10 fruits was taken from each tree, and a composite of these fruits taken for analysis. Individual records for each tree were thereby obtained. In order to eliminate diurnal variations, the sample from one tree of each of the 20 plots was picked and analyzed on the same day. In all cases but one the fruit from the individual trees of each plot was picked on successive days. The Valencia samples of the crop of 1914 were picked on alternate days.

The samples were first weighed and the specific gravity of the fruits taken by immersing them in jars of alcohol of varying specific gravity until the fruit and liquid were in equilibrium, the specific gravity of the liquid being taken as that of the fruit. It was then peeled and ground through a food chopper. The ground sample was placed in a double cheesecloth bag and pressed in a 2-quart fruit press for 20 minutes. The peel, material left in the bags, and juice were weighed; and from these figures the percentage of juice was obtained. All other determinations were

made on the juice, its specific gravity being determined by a spindle and calculated to 15° C. Total sugars, after inversion by Clerget's method, were determined by the Bertrand method, and the sugar calculated as reducing sugars. The acidity was determined by titration with *N/10* alkali with phenolphthalein and the results expressed as anhydrous citric acid. The nitrogen was determined by the Gunning method.

There was some variation in the results obtained from the different trees of a given plot, particularly in the percentage of sugar, but in those cases where notable effects were produced by the fertilizer used, as, for example, the nitrogen content, the variations were nearly always within the limit of experimental error. Table I presents the individual tree records of a plot chosen at random, to show the range of variation. The plot shown is F, fertilized with stable manure.

TABLE I.—*Composition of oranges from individual trees of a single plot*

No.	Specific gravity.	Juice	Sugar.	Acid.	Nitrogen.	Phosphate (P_2O_5).	Potash (K_2O).
		Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
F 282	0.88	37.9	10.74	1.02	0.117	0.052	....
F 283	.87	40.7	10.40	.89	.116	.050	0.21
F 284	.89	39.8	10.46	1.05	.109	.051	.23
F 286	.88	37.3	10.47	.97	.113	.052	.23
F 287	.90	42.1	9.09	.94	.117	.052	.....
F 288	.88	40.1	9.18	.91	.121	.052	.23
Average	.88	39.6	10.06	.96	.115	.052	.23

For the sake of brevity the data have been compiled by plots, the figures presented being merely the averages of the individual tree records obtained. These averages have been assembled with a view to facilitating comparisons of the effect of different fertilizers. In Tables II and III are presented the data from the check plots, those treated with nitrogen, potash, or phosphates only, and the complete-fertilizer plots.

Under the conditions of this experiment, neither potash nor phosphate exercised any effect on the sugar content, but nitrogenous fertilizers, alone or in combination with potash and phosphate, depressed the sugar content. Averaging the figures for sugar in the two crops of each variety, the potash plots show 9.37 per cent, the check plots 9.36 per cent, and the phosphate plots 9.25 per cent. These variations are all within the limits of analytical error. The plots treated with nitrogen alone and those treated with complete fertilizer contained 8.85 per cent and 8.81 per cent, respectively, or about 0.5 per cent less than the other plots. This is a recognizable difference and constant enough to be ascribed to the treatment. It is interesting to note that the effects are greater in the 1915 crops, which were harvested about two months later, than in the

1914 crops. This indicates that nitrogen produced an effect other than that of merely delaying the time of maturity.

The percentage of acid shows the same kind of variation, but in the opposite direction. The check, potash, and phosphate plots contained 0.92 per cent as an average of two crops of each variety, the three being in exact agreement, while the plots treated with nitrogen and a complete fertilizer show 0.94 and 0.96 per cent, respectively. While this is an extremely slight variation, it is interesting to note that it would still further tend to accentuate the lower sugar content in its effect on the quality of the fruit. The specific gravity of the fruit varied slightly, averaging for the four crops as follows: Potash 0.92, phosphate 0.91, check 0.91, nitrogen 0.90, and complete fertilizer 0.90. This confirms the popular contention that potash fertilizers produce a finer fruit and nitrogen a coarser one. The percentage of juice varied with the specific gravity, the averages being 49.2 for potash, 49.2 for controls, 48.2 for phosphate, 47 for nitrogen, and 47.4 for the complete fertilizer.

The quantity of nitrogen present in the juice of the fruit is definitely correlated with the application of nitrogenous fertilizers. In every case the fruit from plots to which nitrogen has been applied shows an increased percentage of nitrogen. This would seem to corroborate the figures on yields, which show nitrogen to be a limiting factor in crop production in this experiment.

TABLE II.—*Composition of navel oranges fertilized differently*

Fertilizer and plot.	Crop of 1914					Crop of 1915.				
	Specific gravity.	Juice	Sugar.	Acid.	Nitrogen.	Specific gravity.	Juice	Sugar.	Acid.	Nitrogen.
		Per cent.	*Per cent.*	*Per cent.*	*Per cent.*		*Per cent*	*Per cent*	*Per cent.*	*Per cent.*
Dried blood, plot C	0.91	47 1	9.28	1 09	0.088	0.89	40.4	10 11	0.98	0.130
Nitrate of soda, plot H	.90	45 5	9 11	1.09	095	.87	38.2	9 26	.94	.137
Dried blood, plot S	.88	44.5	8.86	1.13	.102	.88	40 7	10.05	.99	.117
Average	.90	45 7	9.08	1.10	.095	.88	39 8	9 81	.97	.128
Sulphate of potash, plot D	.92	45.5	9.05	1.08	.076	.91	44.4	10 92	.97	.086
Muriate of potash, plot I	.91	46.9	9.44	1.11	.075	.92	45 0	10.46	.96	.093
Sulphate of potash, plot R	.89	45.9	9 04	1.12	.081	.90	13.9	9.92	.97	.081
Average	.91	46.1	9 34	1 10	077	91	44.4	10 43	.97	.087
Steamed bone, plot E	.90	46.9	9 69	1 01	.082	90	42.9	10.82	.90	.093
Superphosphate, plot J	.90	47 5	8 79	1.08	.081	.90	40 1	10.41	.97	.098
Superphosphate, plot N	.89	47.0	9 00	1.10	.070	.89	41.7	10.12	1.02	.097
Steamed bone, plot P	.90	47.8	9.14	1.13	.082	.89	41 4	10 47	.98	.097
Average	.90	47 3	9.15	1.08	.079	.90	41.5	10 46	.97	.096
Complete fertilizer, plot A	.90	47 3	9.06	1.11	.087	.88	41 7	9.46	.98	.126
Complete fertilizer, plot Q	.89	44.8	9.05	1.13	.102	.88	41.1	9.99	1.00	.124
Average	.90	46 1	9.06	1.12	.095	.88	41.4	9.73	.99	.125
Check, plot B	.91	47.5	9.39	1.06	.072	Missing.				
Check, plot M	.89	46.9	9.04	1.07	.084	.90	43 1	10.23	.98	.096
Check, plot T	.88	45 8	9.47	1 12	.082	.90	43.6	10 80	.92	.087
Average	.89	46.7	9.30	1.08	.079	.90	43.4	10.51	.95	.092

TABLE III.—*Composition of Valencia oranges fertilized differently*

Fertilizer and plot.	Crop of 1914.					Crop of 1915.				
	Specific gravity.	Juice	Sugar.	Acid.	Nitrogen.	Specific gravity.	Juice.	Sugar.	Acid.	Nitrogen.
		Per cent	*Per cent.*	*Per cent.*	*Per cent.*		*Per cent*	*Per cent.*	*Per cent.*	*Per cent.*
Dried blood, plot C	0 92	52.5	8.68	0.97	0.116	0.92	53 6	8 37	0 66	0.128
Nitrate of soda, plot H	.92	52.0	8.40	1.01	.130	.91	52.8	7 01	.74	.139
Dried blood, plot S	.89	47 4	7.84	.97	.124	.91	48.9	9 26	.78	.141
Average	91	50 6	8.31	.98	.123	.91	51.8	8 21	.73	.136
Sulphate of potash, plot D	.94	53 3	9.04	.98	.089	.95	55 3	9.09	.60	.080
Muriate of potash, plot I	92	52 0	8.59	.97	.095	.94	50.7	9 28	.67	094
Sulphate of potash, plot R	.90	49 4	8.22	.96	.107	.92	53 8	8 94	.67	.099
Average	92	51 6	8 62	.97	.097	94	55.3	9 10	.65	.091
Steamed bone, plot E	.91	52.5	8.66	.92	.095	.93	53.3	9 16	.64	.108
Superphosphate, plot J	.91	50.1	8.46	.96	.108	.94	54.8	9.39	.68	.119
Superphosphate, plot N.	.91	51 0	8.24	.90	.089	.93	53.0	8 95	.67	.111
Steamed bone, plot P	90	50 0	7.99	.94	.110	.92	50 8	9 07	.71	.114
Average	.91	50 9	8.34	.93	.101	.93	53.0	9 14	.68	.113
Complete fertilizer, plot A	.93	53 9	8.07	1.01	.124	.92	53 1	7 93	.76	.141
Complete fertilizer, plot Q	.89	47.5	7.84	1.00	.130	91	48 5	9 01	.81	.146
Average	.91	50 7	7.96	1.01	127	.92	50 8	8.47	.74	.144
Check, plot B	.92	53 0	8.89	1 02	.088	.92	55 9	8 58	.65	.071
Check, plot M	91	51 8	8 39	.94	.094	.93	56.1	8 75	.67	.095
Check, plot T	90	49 1	8 26	.99	.104	.94	53 6	10.00	.65	.103
Average	.91	51 3	8.51	.98	.095	.93	55.2	9.11	.66	.090

To show still further the specific effect of the nitrogenous fertilizers, Tables IV and V have been prepared, in which the plots receiving nitrogen, either alone or in any combination, are grouped together and contrasted with all the plots not receiving that element.

TABLE IV.—*Comparison of navel oranges fertilized with and without nitrogen*

CROP OF 1914

Fertilized with nitrogen.						Not fertilized with nitrogen.					
Plot	Specific gravity.	Juice.	Sugar.	Acid.	Nitrogen.	Plot.	Specific gravity.	Juice.	Sugar.	Acid.	Nitrogen.
		P. ct.	*P. ct.*	*P. ct.*	*P. ct*			*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*
A	0.90	47.3	9.06	1.11	0.087	B	0.90	47.3	9.04	1.11	0.087
C	.91	47.1	9.28	1.09	.088	D	.92	45.5	9.65	1.08	.076
F	.90	44.7	9 08	1.13	.088	E	.90	46.9	9.69	1.01	.082
G	.90	46.0	9.01	1.02	.091	I	.91	46.9	9.44	1.11	.075
H	90	45.5	9.11	1.09	.095	J	.90	47.5	8.79	1.08	.081
L	.90	47.2	8.75	1.09	.095	K	.90	47.8	8.98	1.13	.069
O	.89	43.2	9.16	1.14	.096	M	.89	46.9	9.04	1.07	.084
Q	.89	44.8	9.05	1.13	.102	N	.89	47.0	9.00	1.10	.070
S	.88	44.6	8.86	1.13	.102	P	.90	47.8	9.14	1.13	.082
						R	.89	45.9	9.04	1.12	.081
						T	.88	45.8	9.47	1.12	.082
Average	.90	45.6	9.04	1.10	.094	Average.	.90	46.8	9.21	1.10	.079

TABLE IV.—*Comparison of navel oranges fertilized with and without nitrogen*—Contd.

CROP OF 1915

Fertilized with nitrogen.						Not fertilized with nitrogen.					
Plot.	Specific gravity.	Juice.	Sugar.	Acid.	Nitrogen.	Plot.	Specific gravity.	Juice.	Sugar.	Acid.	Nitrogen.
A	0.88	41.7	9.46	0.98	0.126	B	(a)	(a)	(a)	(a)	(a)
C	.89	40.4	10.11	.98	.130	D	0.91	44 4	10.92	0.97	0.086
F	.88	39.6	10.06	.96	.115	E	.90	42.9	10 82	.90	.093
G	.87	38.0	9 62	1.01	.132	I	.92	45.0	10.46	.96	.093
H	.87	38.2	9.26	.94	.137	J	.90	40.1	10.41	.97	.098
L	.88	41.1	9 73	.94	.121	K	.89	43.4	9.98	1.00	.112
O	.87	37.5	10 08	1.01	.097	M	.90	43.1	10 23	.98	.096
Q	.88	41.1	9 99	1.00	.124	N	.89	41 7	10.12	1.02	.097
S	.88	40.7	10 05	.99	.117	P	.89	41 4	10 47	.98	.097
						R	.90	43 9	9 92	.97	.081
						T	.90	43 6	10 80	.92	.087
Average.	.88	39.8	9.82	.98	.122	Average.	.90	43.0	10.41	.96	.094

[a] No analysis because of failure of the crop

TABLE V.—*Comparison of Valencia oranges fertilized with and without nitrogen*

CROP OF 1914

Fertilized with nitrogen.						Not fertilized with nitrogen.					
Plot.	Specific gravity.	Juice	Sugar.	Acid.	Nitrogen.	Plot.	Specific gravity.	Juice.	Sugar	Acid.	Nitrogen.
		P. ct.	*P. ct.*	*P. ct.*	*P. ct*			*P. ct*	*P. ct.*	*P. ct.*	*P. ct.*
A	0.93	53.9	8.07	1.01	0.124	B	0.92	53 0	8.89	1.02	0.088
C	.92	52.5	8.68	.97	.116	D	.94	53 3	9.04	.98	.089
F	.92	51.1	8 27	.98	.117	E	.91	52 5	8.66	.92	.095
G	.92	52.3	8.35	.97	.119	I	.92	52.0	8 59	.97	.095
H	.92	52 0	8 40	1.01	.130	J	.91	50.1	8.46	.96	.108
L	.92	51.6	8.05	1.02	.132	K	.91	51.3	8 15	.92	.097
O	.89	49 5	8.07	.96	.118	M	.91	51.8	8 39	.94	.094
Q	.89	47.5	7.84	1.00	.130	N	.91	51.0	8.24	.90	.089
S	.89	47.4	7.84	.97	.124	P	.90	50.0	7 99	.94	.110
						R	.90	49.4	8.22	.96	.107
						T	.90	49.1	8.26	.99	.104
Average.	.91	50.9	8.17	.99	.123	Average.	.91	51.2	8.44	.95	.098

CROP OF 1915

A	0.92	53.1	7.93	0.76	0.141	B	0 92	55.9	8.58	0.65	0.071
C	.92	53 6	8.37	.66	.128	D	.95	55.3	9.09	.60	.080
F	.92	52.1	8.67	.71	.125	E	.93	53.3	9.16	.64	.108
G	.92	52.4	8.40	.75	.143	I	.94	56.7	9.28	.67	.094
H	.91	52.8	7.01	.74	.139	J	.94	54.8	9 39	.68	.119
L	.92	52 6	7.79	.72	.148	K	.92	53.2	8.19	.69	.108
O	.92	49.9	8.86	.75	.135	M	.93	56.1	8.75	.67	.095
Q	.91	48.5	9.01	.81	.146	N	.93	53.0	8.95	.67	.111
S	.91	49.0	9026	.78	.141	P	.92	50.8	9.07	.71	.114
						R	.92	53.8	8.94	.67	.099
						T	.94	53.6	10.00	.65	.103
Average.	.92	51.6	8.37	.74	.138	Average.	.93	54.2	9.04	.66	.100

The averages of all plots receiving nitrogen agree closely, whether the nitrogen was applied singly or in combination with other fertilizers, as shown by Table VI.

TABLE VI.—*Average composition of oranges when fertilized with nitrogen alone and in combination*

Fertilizer.	Specific gravity.	Juice.	Sugar.	Acid.	Nitrogen.
		Per cent.	*Per cent.*	*Per cent.*	*Per cent.*
Checks	0.91	49.2	9.36	0.92	0.089
Nitrogen alone	.90	47.0	8.85	.94	.121
Nitrogen with other fertilizers	.90	47.0	8.84	.95	.119

It may be concluded, then, that nitrogen exercised a definite influence on the composition of the crop, whether applied with or without other fertilizers.

The average composition with and without potash is shown in Table VII.

TABLE VII.—*Comparison of the averages of all plots receiving potash and those not receiving potash*

Fertilizer.	Specific gravity.	Juice.	Sugar.	Acid	Nitrogen.
		Per cent	*Per cent.*	*Per cent*	*Per cent.*
Check	0.91	49.2	9.36	0.92	0.089
Potash	.91	48.5	9.02	.94	.105
Without potash	.91	47.6	9.12	.93	.105

These averages are somewhat lower than those of the plots receiving potash only. This, however, does not necessarily indicate that the percentages (of sugar, for example) have been increased by the use of potash alone as compared with potash in combination with other fertilizers, but rather that the depressing effect of nitrogen appears in the latter case. This is more clearly shown by contrasting the average of plot L, which received potash and nitrogen, with plot R, which is nearly adjacent and received potash alone. The average percentage of sugar in the fruit from plot L was 8.58, somewhat lower than that of the plots fertilized with nitrogen only, while R showed 9.03 per cent.

On the whole, then, Table VII, when considered in conjunction with Table VI, lends but little support to the view that potash fertilizers materially modify the composition of oranges. A careful study of the data in Tables II and III will likewise show but little effect from phosphate. It is only fair to state, however, that this soil is naturally well supplied with both potash and phosphate, and therefore conclusions should not be drawn with reference to the effects of potash and phosphate fertilizers on the composition of oranges generally.

EFFECT OF SOIL DIFFERENCES

Since the most evident contrast in soil type met with in these experiments occurs in the extreme upper and lower tiers of plots, where the fertilizer treatments have been duplicated, it is of interest to compare the composition of the fruit in the two cases. Such a comparison should throw some light on the question of whether or not the variability of the soil was great enough to vitiate the results in general. As already stated, the soil in the upper tier of plots is lighter in character than that in the lower tier.

The data for the upper and lower tiers of plots have been assembled in Table VIII.

TABLE VIII.—*Composition of oranges from light and heavy soil*

NAVEL ORANGES, 1914

Light soil					Heavy soil				
Plot.[a]	Specific gravity.	Juice.	Sugar.	Acid	Nitrogen.	Plot.[a]	Specific gravity	Juice	Sugar.	Acid	Nitrogen.
		P. ct.	*P. ct.*	*P. ct.*	*P. ct.*			*P. ct*	*P. ct*	*P. ct*	*P. ct.*
A	0.90	47.3	9.06	1.11	0.087	P	0.90	47.8	9.14	1.13	0.082
B	.91	47.5	9.39	1.06	.072	Q	.89	44.8	9.05	1.13	.102
C	.91	47.1	9.28	1.09	.088	R	.89	45.9	9.04	1.12	.081
D	.92	45.5	9.65	1.08	.076	S	.88	44.6	8.86	1.13	.102
E	.90	46.9	9.69	1.01	.082	T	.88	45.8	9.47	1.12	.082
Average	.91	46.9	9.41	1.07	.081	Average.	.89	45.8	9.11	1.13	.090

NAVEL ORANGES, 1915

A	0.88	41.7	9.46	0.98	0.126	P	0.89	41.4	10.47	0.98	0.097
B						Q	.88	41.1	9.99	1.00	124
C	.89	40.4	10.11	.98	.130	R	.90	43.9	9.92	97	.081
D	.91	44.4	10.92	.97	.086	S	.88	40.7	10.05	99	.117
E	90	42.0	10.82	.90	.093	T	.90	43.6	10.80	.92	.087
Average	.90	42.4	10.33	.96	.109	Average	89	42.1	10.25	.97	.101

VALENCIA ORANGES, 1914

A	0.93	53.9	8.07	1.01	0.124	P	0.90	50.0	7.99	0.94	0.110
B	.92	53.0	8.89	1.02	.088	Q	.89	47.5	7.84	1.00	.130
C	.92	52.5	8.68	.97	116	R	.90	49.4	8.22	.96	.107
D	.94	53.3	9.04	.98	.089	S	89	47.4	7.84	.97	.124
E	91	52.5	8.66	.92	.095	T	.90	49.1	8.26	99	104
Average	.92	53.0	8.67	.98	.102	Average	.90	48.7	8.03	.97	.115

VALENCIA ORANGES, 1915

A	0.92	53.1	7.93	0.76	0.141	P	0.92	50.8	9.07	0.71	0.114
B	.92	55.9	8.58	.65	.071	Q	.91	48.5	9.01	.81	.146
C	.92	53.6	8.37	.66	.128	R	.92	53.8	8.94	.67	.099
D	.95	55.3	9.09	.60	.080	S	.91	49.0	9.26	.78	.141
E	.93	53.3	9.16	.64	.108	T	.94	53.6	10.00	65	.103
Average	.93	54.2	8.64	66	.106	Average	.92	51.1	9.26	.72	.121

[a] The fertilizers applied to the different plots are given in Table X.

The average sugar content for the two crops of each variety is 9.19 for the upper tier and 9.16 for the lower, showing very close agreement. In every case, except that of the Valencia crop for 1915, the individual plots of the lower tier (P, Q, R, S, T) show the same characteristics as those of the nitrogen plots in general—that is, the fruit from them had a lower sugar content, a little more acid, a slightly lower specific gravity and percentage of juice, and a higher nitrogen content than that from other plots not fertilized. While the data indicate a somewhat richer soil in the lower tier of plots, the differences are not sufficiently great to affect materially any conclusions that may be drawn from the experiment as a whole.

COMPARISON OF ORANGES FERTILIZED WITH COMMERCIAL FERTILIZER AND ORGANIC MATTER

The plots in the fertilizer experiment have been kept in clean cultivation throughout the experiment. In adjacent plots on the same type of soil are trees of the same age which have received large amounts of stable manure, together with rock phosphate and a leguminous cover crop. The trees on these plots present a thriftier appearance and bear more fruit than the trees in the fertilizer experiment. There is a general impression that the use of a cover crop and organic matter tends to produce oranges of coarser texture and poorer quality. Analyses of fruit from the cover-cropped plots were made along with those from the fertilizer plots. The results obtained do not indicate any great difference, and although some variations appear, the closeness of the agreement is rather striking, as is shown by Table IX.

TABLE IX.—*Composition of oranges fertilized with commercial fertilizer and with organic matter*

Crop.	Fertilized plots					Cover-crop plot.				
	Specific gravity.	Juice.	Sugar.	Acid.	Nitrogen	Specific gravity.	Juice.	Sugar.	Acid.	Nitrogen.
Navel:		*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*		*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
1914.....	0.90	45.7	9.13	1.10	0.087	0.89	45.8	9.15	1.05	0.104
1915.....	.88	41.4	10.12	.98	.108	.86	37.8	9.96	.95	.129
Valencia:										
1914.....	.91	51.1	8.31	.97	.111	.91	47.3	8.51	.92	.128
1915.....	.93	52.9	8.69	.70	.119	.91	50.0	8.44	.79	.135

Two explanations suggest themselves as accounting for the greater quantity of nitrogen found in the fruit from plots to which nitrogen has been applied. (1) In the presence of a greater quantity of available nitrogen in the soil the orange tree is able to absorb this element in excess of its needs. (2) The greater quantity of nitrogen was necessary for the promotion of more normal growth. It seemed rather improbable

that the small quantities being added increased the amount available to the tree to a point where it would be taken up in excess. No direct evidence was obtained, however, since the same quantity was applied in each case.

THE EFFECT OF FERTILIZERS ON THE PHOSPHORIC-ACID AND POTASH CONTENT OF ORANGES

Although this investigation was undertaken primarily to determine whether the fertilizers affected those characteristics of the orange usually considered in connection with its quality, the results obtained in the nitrogen determination suggested that work be undertaken in regard to the absorption of other elements of fertility. Accordingly, the phosphoric-acid content was determined in the Valencia crop of 1914 and the navel crop of 1915. In the 1915 crop the percentage of potash was also determined. Table X presents the results.

TABLE X.—*Effect of various fertilizers on the phosphoric-acid and potash content of Valencia and navel oranges*

Plot.	Fertilizer applied	Per cent of phosphoric acid Valencia oranges, 1914.	Per cent of phosphoric acid navel oranges, 1915.	Per cent of potash navel oranges, 1915.
A	Complete, nitrate of soda, blood, bone, sulphate of potash	0.059	0.051	0.23
B	Check	.066	.....	.....
C	Dried blood	.059	.052	.23
D	Sulphate of potash	.072	.056	.....
E	Steamed bone	.063	.051	.22
F	Stable manure	.056	.052	.23
G	Nitrate of soda, blood, and steamed bone	.055	.052	.24
H	Nitrate of soda	.055	.053	.24
I	Muriate of potash	.061	.052	.....
J	Superphosphate	.058	.052	.23
K	Bone and sulphate of potash	.059	.051	.23
L	Nitrate of soda	.059	.052	.23
M	Check	.060	.052	.23
N	Superphosphate	.062	.053	.23
O	Stable manure and raw phosphate rock	.055	.047	.23
P	Steamed bone	.057	.051	.23
Q	Complete, like A, except superphosphate instead of bone	.057	.050	.23
R	Sulphate of potash	.055	.052	.22
S	Dried blood	.056	.050	.23
T	Check	.059	.051	.23

There was no increase in the amount of either phosphate or potash in the fruit brought about by the quantities applied in this experiment. The averages from those plots receiving fertilizers are almost identical with those not fertilized. This gives further significance to the increase in nitrogen content brought about by nitrogen fertilization and supports the view that nitrogen is the limiting factor in the production of oranges on this soil.

SUMMARY

(1) Nitrogen is the only fertilizer which in this experiment seemed to exercise a specific effect on the composition of oranges.

(2) Applications of nitrogen to the soil resulted in a slightly lower amount of sugar, a somewhat coarser fruit, and a little less juice in the orange.

(3) The effect of nitrogen was the same, whether applied alone, in combination with either potash or phosphoric acid, or both.

(4) The effect of nitrogen was greater in 1915 than in 1914. As the crop was picked about two months later in 1915, it would indicate that some effect other than delayed maturity was caused by the nitrogen.

(5) Comparison with fruit from similar trees grown outside the fertilizer plots shows a fair agreement of composition and quality.

(6) The analyses show a higher percentage of nitrogen from all plots receiving it, while no such effect was obtained with either phosphoric acid or potash.

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TEMPERATURE RELATIONS OF APPLE-ROT FUNGI[1]

By CHARLES BROOKS, *Pathologist*, and J. S. COOLEY, *Assistant Pathologist, Fruit Disease Investigations, Bureau of Plant Industry, United States Department of Agriculture*

INTRODUCTION

The retarding effect of low temperatures on plant activities is a matter of general knowledge, and the principle has had a very general application in the storage of fruits. The better preservation of the fruit at low temperatures is due both to the slowing up of the activities of the fruit itself and to the checking of fungus and bacterial growth. This paper deals particularly with the latter phase of the storage problem. An effort has been made to carry on the investigations in a manner that would make the data of general physiological interest as well as of practical value in fruit storage.

HISTORICAL REVIEW

Eustace[2] made one of the first studies of the storage-rot problem in this country. Several varieties of apples (*Malus sylvestris*) were inoculated with *Alternaria* sp., *Glomerella rufomaculans* (Berk.) Sp. and Von Schr., *Sphaeropsis malorum* Pk., *Penicillium glaucum* Lk., *Sclerotinia fructigena* (Pers.) Schrt., and *Cephalothecium roseum* Cda.; and similar inoculations were made on agar petri plates. After two months at a temperature that varied from 30° to 33° F. and averaged 32° *Penicillium glaucum* had made a good growth on both the agar and the apples and *Alternaria* sp. had made a slight growth on the apples. None of the other fungi had produced any growth, but all of them developed rapidly when removed to a temperature of 70°. In another experiment inoculated fruit and agar plates were placed in a storage room in which the temperature ranged from 35° to 56° and averaged 47°. All of the fungi made a good growth. At a temperature of 48° to 69° the development of the fungi was still more rapid.

Schneider-Orelli[3] made experiments with *Glocosporium herbarum* Lk., *Mucor piriformis* Fisch., *Penicillium glaucum*, *Botrytis cinerea* Pers., *Monilia fructigena* Pers., *Fusarium putrefaciens* Osterw., *Gloeosporium album* Osterw., *Gloeosporium fructigenum* Berk., and *Rhizopus nigricans*

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Ehrbg. He grew the fungi on gelatin in petri plates at temperatures of 0°, 4.5°, 9.5°, 14°, and 18° C. At the end of 35 days all but the last two of these fungi had made a measurable growth at 0°, *Botrytis cinerea* making the most vigorous growth. At 4.5° *Rhizopus nigricans* and *Gloeosporium fructigenum* had produced small colonies at the end of 35 days.

Penicillium glaucum, *Botrytis cinerea*, *Monilia fructigena*, and *Gloeosporium album* were inoculated into apples and the fruit stored in one case at 4.5° and in another at 14° C. The diameter of the rots produced at the end of two and three weeks is given. The first three fungi made a fair growth at the lower temperature.

In a later paper Schneider-Orelli [1] reported temperature studies on different species or strains of *Gloeosporium fructigenum*. He found that the European form had lower optimum, maximum, and minimum temperatures than the American form. At 5° C. the European form, when grown in gelatin in petri plates, produced a colony 0.4 cm. in diameter in 12 days and a colony 3.7 cm. in diameter in 35 days, while the American form had made no growth at the end of 35 days.

Edgerton [2] made a study of the behavior of various American species and strains of Glomerella. He found that there were two different strains of Glomerella on the apple, a rapid-growing southern form and a slow-growing northern form. The former had an optimum temperature of 27° to 29° C. and a maximum temperature above 37.5°, produced scanty aerial mycelium in culture, formed cankers on apple limbs, besides rotting the fruit, and very commonly produced perithecia on the host and in culture. The latter had an optimum temperature of 24° to 25° and a maximum temperature of 34° to 35°, produced a greater abundance of white aerial mycelium in culture, and apparently did not produce cankers on apple limbs, though it rotted the fruit. He thought the former should be called "*Glomerella cingulata*" and that the latter should retain the name "*Gloeosporium fructigenum*" given by Berkeley. He considered the latter fungus probably identical with the European one.

Ames [3] determined the germination and growth of *Monilia fructigena*, *Penicillium digitatum* (Fr.) Sacc., *Rhizopus nigricans* Ehrenb., *Glomerella rufomaculans*, and *Cephalothecium roseum* at various temperatures. The germination tests were made by means of Van Tieghem cell drop cultures. At 1° C. only the first two of these fungi germinated within 245 days, while at 3° to 4° all but *Cephalothecium roseum* germinated within 200 days. The growth of the fungi was tested on bean agar, and in this series *Penicillium glaucum* was substituted for *P. digitatum*. None of the fungi except *P. glaucum* made any growth at a temperature of 1° to 2° and

[1] Schneider-Orelli, Otto. Op cit., 1912.

[2] Edgerton, C. W. The physiology and development of some anthracnoses. *In* Bot. Gaz., v. 45, no. 6, p. 393, 402–403. 1908.

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[3] Ames, Adeline. The temperature relations of some fungi causing storage rots. *In* Phytopathology, v. 5, no. 1, p. 11–19. 1915.

this produced but very small colonies. At a temperature of 3° to 4° *Monilia fructigena* made a scant development, and at 9° to 10° all of the fungi made some growth, the species of Monilia and Penicillium both fruiting within 11 days.

EXPERIMENTAL INVESTIGATIONS

APPARATUS

The experiments reported in this paper were carried out in refrigerator boxes constructed especially for the work. The low temperatures were obtained by means of a sulphur-dioxid refrigerating machine. This machine was stopped and started automatically by means of a tumbler switch and a 3-step starter and the brine thus kept within a range of 2° C. In the cooler months it was kept at a temperature of −8° to −6°, but in the warmer months it was necessary to carry it one or two degrees colder in order to obtain the required refrigeration. In testing out regulators it was found that a short bimetallic form was entirely unsatisfactory in humid weather, as the moisture condensed on the cold metal at the contact points and caused considerable trouble in the operation of the machine. Later, a regulator was used in which the points of contact were placed a long distance from the brine and the make-and-break brought about by means of a column of mercury under pressure; this was found entirely satisfactory. The brine was circulated through the pipes in the boxes by means of a rotary pump which was kept in operation all the time. Different amounts of brine were thrown into the different boxes by means of control valves on the pipes at the entrance to the boxes, thus making it possible to maintain a different temperature in each box.

The walls of the boxes were 5¾ inches thick, sheet cork and heavy paper being used as insulating material. A galvanized-iron box was placed inside the wooden box. On the sides and bottom there was a 3-inch air space between this inner metal box and the outer box, while at the top there was a space of less than an inch between the metal box and the brine pipes. The inner box was 2 feet high and approximately 3 feet square. The air was circulated in each double box by means of blower fans that were operated constantly, one fanning the air over the brine pipes and through the air space between the two boxes, and the other stirring the air of the inside box. The air from this latter fan was carried across the top of the box by means of a special duct and discharged on the opposite side, thus insuring a complete circulation. A closely fitting slide door in the metal box made it possible to circulate the air from over the brine pipes directly through the inner box if quick cooling was desired. The temperature of the air space between the boxes was always kept a few degrees lower than that desired in the inner box, and the latter brought to the required temperature by the use of electric lamps. Since the brine was kept at a practically constant tem-

perature and was distributed to the different boxes according to the cooling required, only a small amount of heating was necessary to maintain the desired temperature in the inner boxes, two 60-watt electric lamps being sufficient in any one box. These lamps were thrown on and off by means of ordinary telegraphic relays operated by mercury regulators. The lamps were placed in the duct that carried the air across the inner box, thus preventing local heating and also the access of light to the fruit.

The apparatus as described gave very satisfactory results. The circulation of the air was sufficient to maintain uniform temperatures throughout the box and the regulation sufficiently delicate to secure practically straight lines on the thermograph charts. The use of the inner box and the double circulation of air made it possible to maintain higher humidities than could have been done if the air that circulated over the fruit had been exposed to the drying effects of the brine pipes.

In the 5° compartment no inner box was used; and although the air was stirred with a blower fan, the top of the box was about one degree warmer than the bottom. The temperature was regulated as with the other boxes and the lamps were inclosed so as to prevent lighting the compartment.

For obtaining the 30° temperature mentioned in the experiments, an ordinary water-jacket incubator with electric heater was used. The air was not stirred and was about a half degree too warm in the top of the chamber and a half degree too cold in the bottom.

FUNGI

The following fungi were used in the experiments: *Alternaria* sp., *Botrytis cinerea, Cephalothecium roseum, Fusarium radicicola* Wollenw., *Glomerella cingulata* (Atk.) S. and S., *Neofabraea malicorticis* (Cord.) Jackson, *Penicillium expansum* (Lk.) Thom, *Pestalozzia funerea* Desm., *Sclerotinia cinerea* (Bon) Schroeter, *Sphaeropsis malorum, Trichoderma* sp., and *Volutella fructi* S. and H.

They were obtained from decayed fruit collected in various parts of the United States, and were selected as the most virulent organisms from a much larger number that were found capable of producing more or less decay on ripe pome fruits. Each fungus was tried on various culture media to find a substance upon which it would make a good growth and good spore production. Most of the fungi were kept in culture on corn-meal agar. *Fusarium radicicola* was usually grown on potato agar and *Penicillium expansum* on either apple or potato agar. Only cultures that appeared to be in a vigorous condition were used. The inoculations were in most cases made with spores, but with fungi such as *Sphaeropsis malorum* that did not fruit readily in culture, mycelium was sometimes used. So far as observed, no contrasts were seen between the results with spore inoculations and those with mycelium.

INOCULATIONS ON FRUIT

Great care was taken in selecting the fruit for inoculation, as it was found by preliminary experiments that the rapidity of the rot depended greatly upon the variety and maturity of the apples. Only fresh, crisp fruit was used, and the apples for a particular experiment were all of the same lot. Uniformity was obtained by selecting seven apples (or as many as there were temperatures) that were similar in size and degree of maturity and distributing these one each in the seven moist chambers that were to be placed at the different temperatures, and repeating the process until the desired number of apples was obtained. Except where otherwise stated, four apples were used at each temperature in each set of experiments.

Care was taken to have the fruit in as sterile a condition as possible. The fleshy part of the stem next to the absciss-layer was found especially subject to infection by various fungi, and in order to eliminate the possibility of contamination from this source the apple stems were always clipped off close to the flesh of the fruit as the first step in preparing for inoculation. The fruit was then washed in soap and water, and this was followed by immersion in mercuric chlorid (1: 1,000) for three minutes, and, finally, the fruit was given a thorough rinsing in sterile water. Inoculations were made by forcing the spores or mycelium down into the flesh of the fruit by means of a platinum needle. The fruit was stored in sterile moist chambers and sufficient moist filter paper added to keep the air in a practically saturated condition. The fruit was cooled to the desired temperatures within a few hours after storage, even in the coldest chambers. Notes were usually taken on the fruit at intervals of one week. The records were made as rapidly as possible, so that the fruit was kept out of the particular box only for a few minutes. At the lower temperatures the notes could be taken still more rapidly in the early stages of the experiment, as the results were usually entirely negative.

The culture of *Sclerotinia cinerea* used in the first set of experiments (see fig. 1) was from a peach plum from Wenatchee, Washington; one of those used in the second experiments (see fig. 2 and 3) was from a prune from Vancouver, Washington, and the other from an apple from West Virginia. The characteristics of the different cultures indicated that *S. cinerea* had been isolated in each case, but the fungus from apples proved a more vigorous rot organism than the others.

With most of the fungi the various inoculations started off together; but with those that were scarcely able to attack the apple, such as the species of Alternaria, Cephalothecium, Fusarium, Pestalozzia, and Trichoderma, there was sometimes considerable difference in the time of starting of different inoculations under the same conditions, especially as the minimum temperature for the particular fungus was approached. In such cases all of the inoculations that were counted at the last of the

experiment in obtaining the average rot were also counted at the first, even though the record was zero.

The comparative behavior of the different fungi is shown in figures 1, 2, and 3. The base line shows the temperature in degrees centigrade; [1] the perpendicular the diameter of the rots in millimeters. The curves are based on the average weekly increase in the diameter. The results given in figure 1 were obtained from inoculations made on March 27, 1915; those of figures 2 and 3 from inoculations made on January 21, 1916.

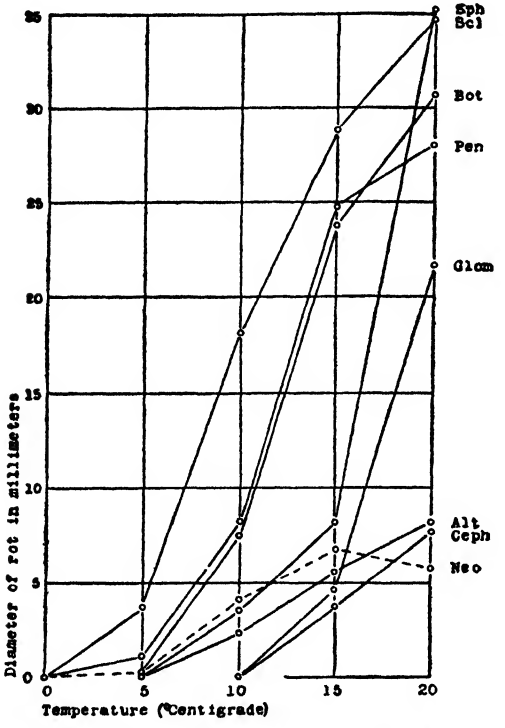


FIG. 1.—Graph showing the development of rot on Ben Davis and York Imperial apples. The curves show the average weekly increase in diameter and are based on records taken two weeks after inoculation.[2]

In the first series of experiments the moist chambers were placed at once at the temperatures desired; in the second they were allowed to remain at 20° for 24 hours before removal to the storage temperatures. A study of the curves will show that some of the rots started off more rapidly at 0° and 5° in the second series of experiments than in the first, but different varieties were used in the two sets of experiments, making it impossible to attribute the contrast entirely to difference in time of storage. The effect of delayed storage will be discussed later in the paper.

It may be noted that some of the curves in figures 2 and 3 are relatively high at 5°. As has already been mentioned, the top of the 5° chamber was 1° warmer than the bottom. During the last experiment this box was used for the storage of other fruit, sometimes resulting in the placing of some of the experimental fruit in close proximity to warm

[1] Temperature equivalents:

°C.	°F.	°C.	°F.	°C.	°F.
30	86	15	59	5	41
25	77	10	50	0	32
20	68				

[2] Abbreviations used in text figures: Alt.=*Alternaria* sp.; Bot.=*Botrytis cinerea*; Ceph.=*Cephalothecium roseum*; Fus.=*Fusarium radicicola*; Glo.=*Glomerella cingulata*; Neo.=*Neofabraea malicorticus*; Pen.=*Penicillium expansum*; Pes.=*Pestalozzia funerea*; Pho.=*Phoma pomi*; Scl.=*Sclerotinia cinerea*; Sph.=*Sphaeropsis malorum*; Tri.=*Trichoderma* sp.: Vol.=*Volutella fructi*.

packages and in the crowding of some of it nearer to the top of the chamber. While the thermograph records did not indicate any error, it is probable that in the last experiment some of the fruit in the 5° chamber was exposed to a temperature about 1° warmer than indicated.

The behavior of the more virulent rot fungi has been practically the same on one variety of apples as on another, but with the weaker organisms the variety of apples has had an important modifying effect upon the temperature results. *Cephalothecium roseum* produced a small amount of rot on the Yellow Newtown at 5°, but was unable to attack the Winesap, York Imperial, and Ben Davis at this temperature or to produce any significant growth on these varieties at 10°. *Fusarium radicicola* was able to rot Newtowns at a lower temperature than Winesap. *Trichoderma* sp. rotted the York Imperial at 20° but did not rot the Ben Davis.

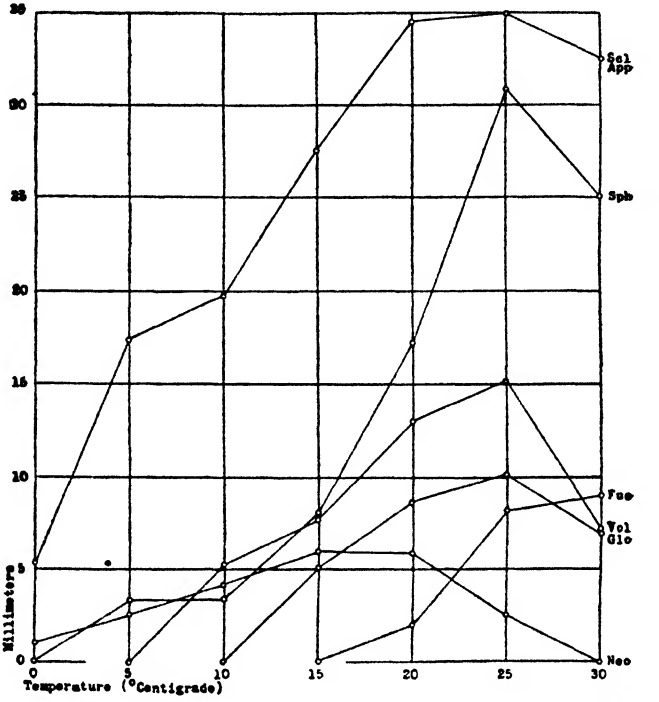


FIG 2.—Graph showing the development of rot on Yellow Newtown apples. The curves show the average weekly increase in diameter. With *Sclerotinia cinerea*, *Sphaeropsis malorum*, and *Volutella fructi* they are based on records made two weeks after inoculation; with the other fungi on records made three weeks after inoculation.

The apparent difference in susceptibility of varieties may have been partly due to differences in ripeness of the fruit, as there seemed to be no satisfactory way of determining the comparative maturity of the different varieties. It was repeatedly observed that a slight increase in the ripeness of apples of a particular variety caused a definite increase in susceptibility to rot. This modifying effect of maturity of the fruit was particularly noticeable as the minimum temperature for the fungus was approached. In this connection it may be mentioned that in cases where fungi had apparently lost some of their rot-producing power as a result of long culturing in the laboratory this attenuation was particularly evident at the lower temperatures.

The contrasts between the temperature responses of the different fungi are very striking. *Sclerotinia cinerea* is the most rapid rot-producing organism tested and is less inhibited by cold than most of the other fungi. *Sphaeropsis malorum* is in general more rapid than *Penicillium expansum;* but the former is practically inhibited at 0°, while the latter can make little growth at 30°. The low optimum and slow rate of growth with *Neofabraea malicorticis* are particularly interesting.

The contrasting results at the different temperatures are shown in graphic manner in Plates 2 and 3.

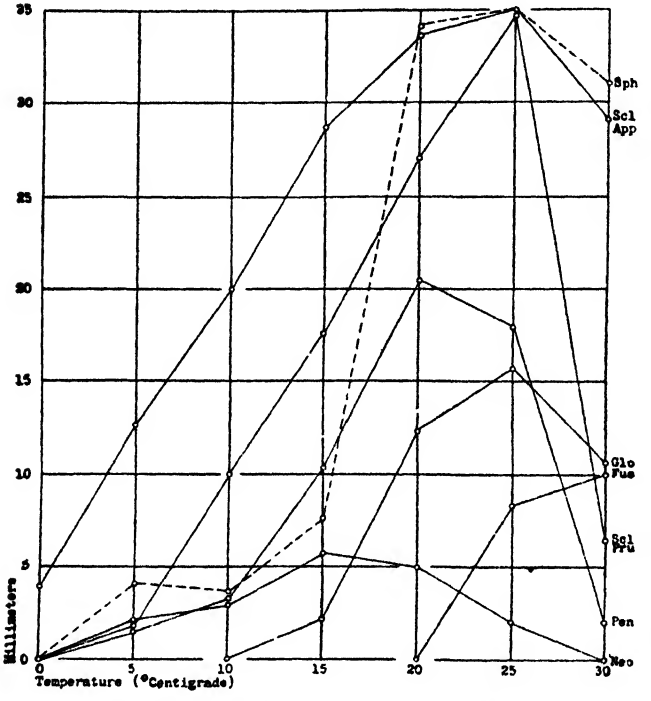


FIG. 3.—Graph showing the development of rot on Winesap apples. The curves show the average weekly increase in diameter. With *Sclerotinia cinerea, Sphaeropsis malorum,* and *Volutella fructi* they are based on records made two weeks after inoculation; with the other fungi on records made three weeks after inoculation.

In figures 1, 2, and 3 the diameter of the rot is taken as the basis of comparison. This does not give the real contrast in size of rot or in the amount of work done by the fungus. A comparison of the temperature effects on a chemical or physiological basis would require that the volume of the rot be considered rather than the diameter. Similar volumes have the same ratio to each other as the cubes of their like dimensions, but unfortunately the masses of rotted tissue do not have similar shapes at the different stages in their development. The fungus can not spread outward, and it usually spreads laterally more rapidly than it does toward the core, thus making the area of the rot a fairly close index of its volume. In figure 4 the curves are plotted on the basis of the average rot areas at the different temperatures. It will be noted that in most cases the increase in growth with a 10° rise in temperature comes within the range of the Van't Hoff law; but there are several striking exceptions, *Sphaeropsis malorum* producing a rot area 24 times

as great at 25° as at 15° and *Penicillium expansum* showing a similar contrast between 10° and 20°. It was also found that Botrytis produced a rot area 25 times as great at 20° as at 10° on the Ben Davis and 57 times as great on the York Imperial.

The progressive development of the rots is shown in figures 5 to 12. The curves are based on the diameter of the rot at the various temperatures after the number of weeks indicated. The temperatures are given on the base line and the diameter of the rots in millimeters on the perpendiculars.

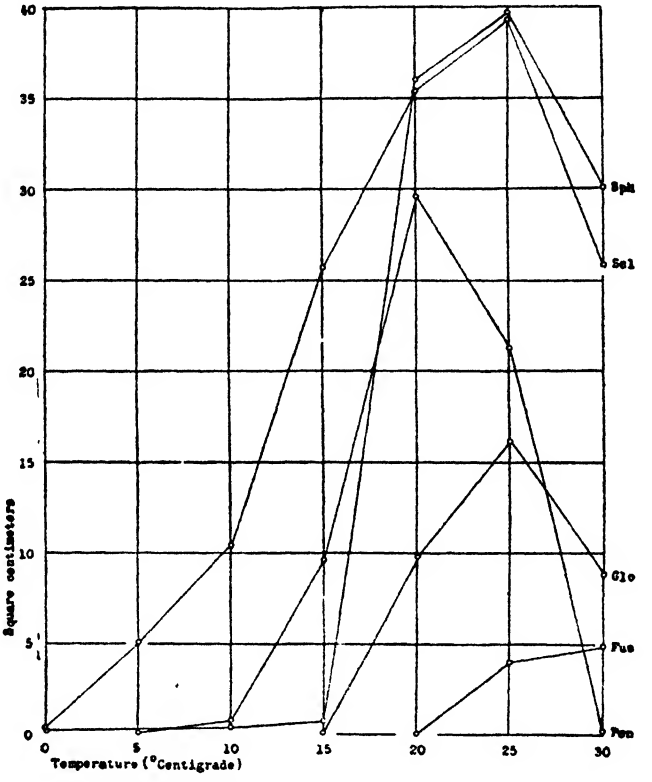


FIG. 4.—Graph showing the development of rot on Winesap apples based on the area instead of the diameter of the rot.

It will be observed that in general the rate of rotting increases with time. In some cases there appear to be exceptions to this rule, but it should be remembered that the curves are plotted on the basis of diameter and not volume, thus giving a decreased value to the growth rate in the larger rots. It is of particular interest to note that the conditions for rapid growth are acquired much more quickly at higher temperatures than at the lower ones. At 20° and 25° most of the fungi became active rot-producing agencies within a week, but as the temperatures became lower, longer and longer periods of incubation were required. When the fungi had once become well established at the lower temperatures, however, the rapidity of the rotting was often most surprising. Good examples of this necessity of incubation may be seen by contrasting the growth of *Sphaeropsis malorum* at 15° and 20° during the first week with that of the second, the growth of *Sclerotinia cinerea* at 0° and 5° during the same two weeks, or the growth of *Penicillium expansum* at 0° and 5° in the first five weeks as compared with that of the last three.

The minimum temperature for some of the fungi seems to be practically absolute, but for others it varies with the length of time the experiment has run. *Fusarium radicicola* was unable to develop at 15° and *Glomerella cingulata* at 10° even after long periods of storage. *Neofabraea malicorticis* and *Penicillium expansum* had produced no rot at 10°

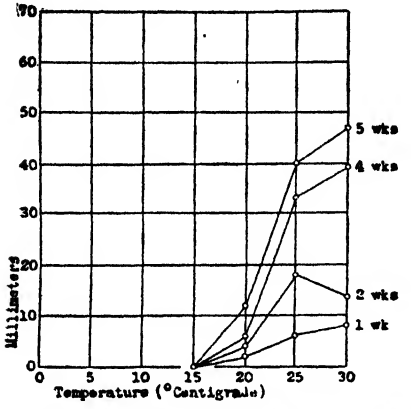


FIG. 5.—Graph showing the development of the rot caused by *Fusarium radicicola* on Yellow Newtown apples.

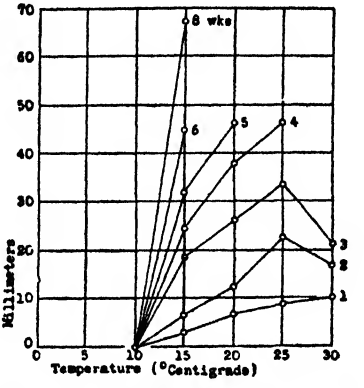


FIG. 6.—Graph showing the development of the rot caused by *Glomerella cingulata* on Yellow Newtown apples.

at the end of two weeks nor *Sphaeropsis malorium* at 15° at the end of one week, but later all made a fair growth at 0°. At the end of two weeks *Alternaria sp.*, *Botrytis cinerea*, and *Volutella fructi* had made no evident growth at 5°, but later developed at 0°. *Sclerotinia cinerea* was the most active at low temperatures of any of the fungi tested, making

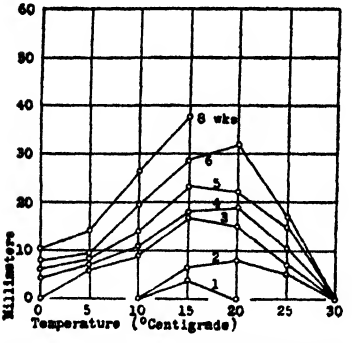


FIG. 7.—Graph showing the development of the rot caused by *Neofabraea malicorticis* on Winesap apples.

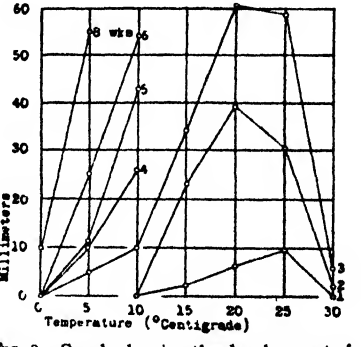


FIG. 8.—Graph showing the development of the rot caused by *Penicillium expansum* on Winesap apples.

a fair growth at 0° by the end of the second week. Further data on the behavior of the various fungi at 0° will be given under the heading of commercial cold storage.

Fusarium radicicola had an optimum at 30° or above; *Glomerella cingulata*, *Sphaeropsis malorum*, *Sclerotinia cinerea*, and *Volutella fructi*

at 25°; *Penicillium expansum* at 20°; and *Neofabraea malicorticis* at 15°. It is interesting to note that the highest summer temperatures are unfavorable to most of the vigorous rot organisms. The fact appears to explain numerous failures that the writers have had with fruit inoculations made in the hottest weeks of the summer. The graphs show that high

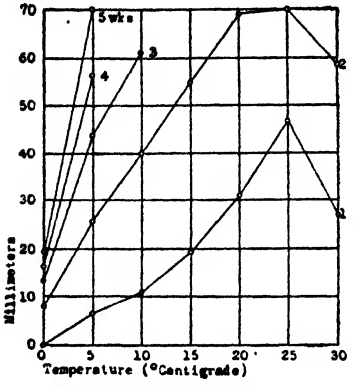


FIG. 9.—Graph showing the development of the rot caused by *Sclerotinia cinerea* (apple) on Winesap apples.

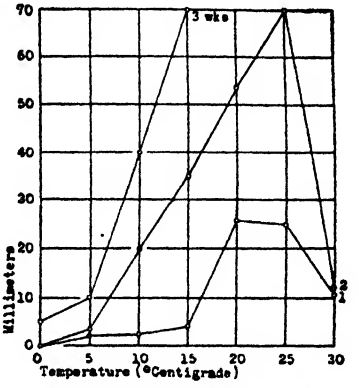


FIG. 10.—Graph showing the development of the rot caused by *Sclerotinia cinerea* (prune) on Winesap apples.

temperatures had a greater retarding effect upon *Neofabraea malicorticis* than upon any of the other fungi. A further illustration of this was found in the removal of inoculated fruit from low temperatures to higher ones. When apples on which *Sphaeropsis malorum*, *Penicillium expansum*,

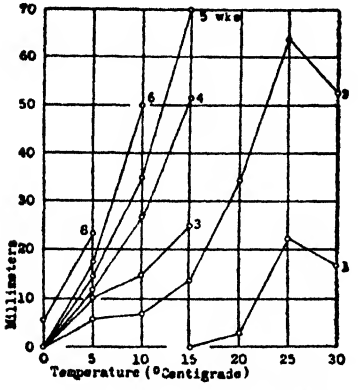


FIG. 11.—Graph showing the development of the rot caused by *Sphaeropsis malorum* on Yellow Newtown apples.

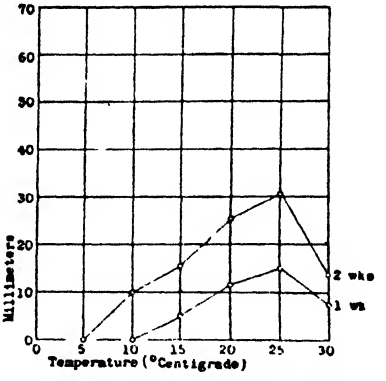


FIG. 12.—Graph showing the development of the rot caused by *Volutella fructi* on Yellow Newtown apples.

and *Neofabraea malicorticis* had produced small rot areas at 0° were removed to laboratory temperature (about 25°), the first two fungi rotted the fruit all the more rapidly at the higher temperature, but the growth of the last named was apparently entirely checked.

The temperatures used were not high enough to determine the maximum for most of the fungi. *Neofabraea malicorticis* refused to grow at 30° and *Penicillium expansum* made but a scant growth at that temperature.

ORCHARD-INFECTED FRUIT

Experiments were also made with *Sphaeropsis malorum* and *Glomerella cingulata*, using rots that were produced by natural infection instead of by artificial inoculation. The condition of the apples at the beginning of the experiment is shown in Plate 1. The rots at this time were mere specks, the largest being only a few millimeters in diameter, and most of them much smaller. The records are based on the increase in the diameter of the rot, the original diameter being subtracted where the initial rot was of measurable size. The blackrot experiments were made on Grimes Golden and Northwestern Greening apples. The results were so uniform with the two varieties that one summary is given for both. The bitter-rot experiments were on Ben Davis apples from Virginia. Part of the apples were placed in large moist chambers, the others in wire baskets. About a peck of apples was used at each temperature for the open package and about half as many in the closed moist chambers. The air in the moist chambers was kept practically saturated. The apples in the open packages were exposed in air that ranged from 50 to 70 per cent relative humidity. The results are given in Tables I and II.

TABLE I.—*Results of natural infection with Sphaeropsis malorum—Experiment started on September 13, 1915*

Temperature.	Container.	Diameter of rot (in millimeters) after—		
		10 days.	24 days.	38 days.
C				
20 to 26, averaging 21	Open basket	23.7	23.7	.....
	Closed chamber	27.2	41.5	.....
15	Open basket	6.6	24.1	.....
	Closed chamber	7.8	27.5	30.0
10	Open basket	1.1	5.5	14.4
0	Open basket	4.2	7.8	13.1
	Closed chamber	0	8	10.5

TABLE II.—*Results of natural infection with Glomerella cingulata*

Temperature.	Container.	Experiment started on September 8. Diameter of rot in millimeters after—				Experiment started on September 28. Diameter of rot in millimeters after—			
		15 days.	29 days.	36 days	43 days	9 days	16 days	23 days.	37 days.
°C.									
39[a]	Closed chamber					4.4			
35[a]	do					9.9	15.5		
28[a]	do					6.0	16.3		
20 to 26, averaging 22.	Open basket	28.7				13.6	24.3		
	Closed chamber					7.1	25.2		
15	Open basket	3.6	9.7	18.0	28.0				
	Closed chamber	0	20.5	31.5	42.0	3.7	7.8		
10	Open basket	0	2.2		6.2				
	Closed chamber	0	4.2		8.7	0	0	0.9	4.7
5	Open basket	0	0		0				
	Closed chamber	0	0		0	0	0	0	0
0	Open basket	0	0		0				
	Closed chamber	0	0		0	0	0	0	0

[a] The three higher temperatures were not constant. At these temperatures the apples soon broke down internally, making it difficult to obtain reliable data.

In the later stages of the experiment the development of the rots appeared to be more rapid in the moist chambers than in the open packages. This was probably due to the fact that the exposed apples became considerably withered as the experiment proceeded and were probably not so easily broken down by the rot fungi.

The results with *Sphaeropsis malorum* are in close agreement with those obtained in the artificial inoculation experiments. This was also true of *Glomerella cingulata*, with the exception that there was no growth at 10° with the artificial inoculations while a slight growth was obtained at that temperature with natural infections. The development at this temperature, however, occurred only with rots that were several millimeters in diameter at the beginning of the experiment.

After two months' exposure to temperatures of 0°, 5°, or 10° infections of *G. cingulata* developed rapidly when the fruit was removed to a temperature of 25°.

APPLE ROTS IN COMMERCIAL COLD STORAGE

Experiments were also made with apples in a commercial cold-storage plant, in which the temperature was held at 0° C. (32° F.). The apples (from the Shenandoah Valley of Virginia) were gathered on October 6, were received in Washington on October 16, and were inoculated on October 19. They were not washed, sterilized, or treated in any way before inoculation. The stems were not recmoved, as was the case in other inoculation experiments. Fruit was selected that was of uniform size and quality and apparently in perfect condition. Ten York Imperial and ten Arkansas apples were inoculated with each fungus by

means of needle punctures. A like number of each variety of apples were allowed to stand for a few minutes in water that had been abundantly inoculated with spores or mycelium from cultures of the particular fungus. The fruit was thus well covered with possible sources of infection. The treated apples were divided into two lots, placed in paper bags, and packed in the middle of barrels with other apples around them. Half of the apples were taken immediately to cold storage, while the other half were kept in the laboratory for one week and then taken to cold storage. They were removed from storage on February 28, and notes were taken immediately. The results are given in Table III.

TABLE III.—*Results of the development of rot on Arkansas and York Imperial apples in commercial cold storage. Inoculations made on October 19; notes taken on February 28*

Fungus.	Average diameter of rot (in millimeters).							
	Inoculated by puncture.				Washed in spore suspensions.			
	Immediate storage.		Storage delayed 7 days.		Immediate storage.		Storage delayed 7 days.	
	Arkansas.	York Imperial.	Arkansas.	York Imperial.	Arkansas.	York Imperial.	Arkansas.	York Imperial.
Alternaria sp. 399	0	0	6	6	0	0	0	0
Alternaria sp. 365	0	0	0	0	0	0	0	0
Aspergillus niger	0	0	23	20	0	0	0	0
Botrytis cinerea	0	0	0	0	0	0	0	0
Cephalothecium roseum	6	7	8	8	0	0	0	0
Fusarium radicicola	0	0	7	35	0	0	0	0
Glomerella cingulata	0	0	9	21	0	0	0	0
Mucor stolonifer	0	0	0	0	0	0	0	(a)
Neofabraea malicorticis	12	15	12	18	0	0	0	0
Penicillium expansum	0	0	3	57	0	0	(b)	0
Sphaeropsis malorum	5	16	25	26	0	0	0	0
Sclerotinia cinerea	75	72	75	75	0	0	0	0
Volutella fructi	0	0	6	7	0	0	0	0

[a] One 45, others 0. [b] One 75, others 0.

The contrasts between immediate and delayed storage are quite striking. The species of Aspergillus, Fusarium, Glomerella, Volutella, Alternaria, and Penicillium produced no rot when the fruit was immediately stored at 0°; while, when storage was delayed for seven days, all produced more or less decay. With the first three of these probably all or nearly all of the rot was produced before the fruit was cooled. This may have been true also of *Alternaria* sp. and *Volutella fructi*, but could not have been the case with *Penicillium expansum* on York Imperial apples, as the experiments already reported show that it is not able to produce rots 57 mm. in diameter in one week. The fact that such an important storage-rot as *P. expansum* failed to start when placed at 0°

but continued to grow when given a start at a higher temperature emphasizes the importance of immediate storage. It is interesting to note how little effect the delay in storage had upon *Cephalothecium roseum* and *Neofabraea malicorticis*. This is in agreement with the temperature responses shown in figures 2 and 7. The growth of *Sphaeropsis malorum* was delayed by immediate storage, and this was probably true of *Sclerotinia cinerea* also; but with the latter fungus no contrast was evident at the end of the experiment, as the fruit was practically completely rotten in the immediate as well as in the delayed storage.

It should be noted that the species of Alternaria, Botrytis, Penicillium, and Volutella used produced no rot when stored immediately at commercial cold-storage temperature, while all have caused more or less rot at 0° in laboratory experiments, *Botrytis cinerea* and *Penicillium expansum* producing rots 1 or 2 cm. in diameter in less than five weeks. It is possible that the air in the cold-storage plant sometimes dropped slightly below 0° C. and that the explanation of the above contrast is to be found in differences in temperature, but the writers are of the opinion that it was due to differences in the maturity of the fruit; as the apples used in the commercial cold-storage experiments were much greener than any of those used in the laboratory tests. The importance of maturity of fruit in determining the amount of rot at low temperatures and the minimum temperature for the fungus has already been pointed out.

It is interesting to note the almost entire absence of rot in the case of the apples washed in spore suspensions, only two apples out of the total of 140 becoming infected at all and these two specimens occurring in the case of delayed storage. The results strongly emphasize the importance of avoiding punctures and other injuries on fruit that is to be held in storage.

TEMPERATURE EXPERIMENTS ON CULTURE MEDIA

The various rot fungi were also tested at different temperatures on several different culture media. The behavior of the fungi on the fruit is of special interest because it is on this medium that they become of economic importance; but the apple is a living organism and when fruit is kept in storage for any length of time, its condition at a high temperature will become very different from that of similar fruit kept at low temperatures. Consequently, in determining the behavior of fungi at different temperatures one must consider two living organisms, the parasite and the host, the former usually being favored by high temperatures and the latter being continually weakened by them. Culture media probably remains practically constant at the different temperatures used and therefore furnishes a fairer test of the temperature responses of the different fungi. It has been difficult, however, to get one medium which was satisfactory for the growth of all the fungi.

FLASK EXPERIMENTS.—In one experiment the fungi were grown on liquid media in 100 c. c. Erlenmeyer flasks. About 50 c. c. of liquid were poured into each flask. Dox's solution plus 2 per cent of dextrose was used with all the fungi except the species of Glomerella and Neofabraea. These did not grow well on this solution, so apple juice about +5 was used as a substitute. The flasks were placed at the particular temperatures immediately after inoculation. The experiment was started on April 16 and was concluded two weeks later. The fungus growth was filtered out by means of filter papers that had previously been dried and accurately weighed. After the fungus mass had been dried to constant weight, a second weighing was taken, the difference between the two weighings representing the dry weight of the fungus. The results are shown in figure 13.

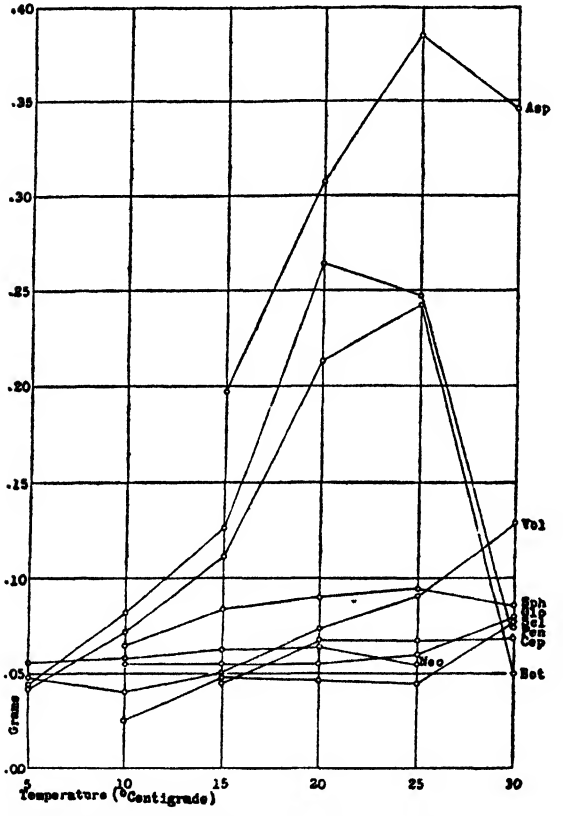


FIG. 13.—Graph showing the growth of apple-rot fungi in liquid media

The growth of some of the fungi was rather poor on the medium used, and in such cases the temperature response was small. It is interesting to note that *Sclerotinia cinerea*, *Glomerella cingulata*, and *Sphaeropsis malorum*, fungi which have given very decided temperature contrasts in other experiments, have in this case given practically none. *Neofabraea malicorticis* has also given little contrast but did not show great sensitiveness to temperature in the experiments on fruit. With the species of Botrytis, Penicillium, and Aspergillus used there was a very decided drop in growth between 25° and 30°. *Botrytis cinerea* and *Aspergillus niger* had an optimum at 25°, while the optimum for *Penicillium expansum* was 20° instead of 25° as in the experiments on fruit. None of the fungi made enough growth at 0° to produce a measurable quantity of mycelium. With many of them there was no evident growth. All of these, however, produced a vigorous growth when later removed to warmer temperatures.

PETRI-DISH EXPERIMENT.—It was found much more satisfactory to test the growth of the fungi on agar in petri dishes than by the use of flasks. Growth was more uniform, more easliy measured, and the results apparently were more reliable. In these experiments corn-meal agar was used for all of the fungi. The culture medium was poured into the dishes, and inoculations were made near the center of the plates as soon as the agar had cooled. Plates were made up in duplicates in all cases. They were allowed to stand in the laboratory for 24 hours after inoculation had been made and were then placed at the various temperatures indicated. In the first series of experiments no measurements of the colonies were taken at the end of this first 24 hours, but records were made after 4, 6, and 17 days. Figure 24 shows the results obtained at the end of 6 days after inoculation. In the second series of experiments measurements were taken at the end of the 24 hours at laboratory temperature and later measurements at the times indicated. The rate of development of the various fungi in this experiment is shown in figures 14 to 23. The graphs show the increase in the diameter of the colonies at the end of 2, 4, 6, 8, 18, and 34 days from the time of placing the cultures at the given temperatures. The comparative temperature responses of the different fungi is brought out in figure 25. In this figure the curves show the average daily increase in diameter after the end of the first day in storage. By not including the growth made in the day at laboratory temperature nor the growth in the first day at the storage temperatures it was thought that a more accurate record of the temperature responses of the fungi would be obtained. The base line represents temperature in all cases, and the perpendicular the diameter of the growth in millimeters.

A study of figures 14 to 23 as compared with figures 4 to 12 makes it evident that the rate of growth did not increase with time in the petri-dish experiments as it did in the experiments with apples, the fungi, with the exception of *Sphaeropsis malorum*, making as great increase in growth at both low and high temperatures during the first days of the experiment as they did during the later ones.

In comparing the growth on apples with that on corn-meal agar it will also be noted that in the latter case fewer of the fungi show a tendency to lower the minimum with the longer periods of time. This is partly accounted for by the fact that more of the fungi grew at the lower temperatures on the corn meal from the beginning of the experiment. *Fusarium radicicola* had made no evident growth on apples at 15° after five weeks nor *Glomerella cingulata* on apples at 10° after eight weeks, but both grew on corn-meal agar at 5° from the first. *Neofabraea malicorticis* and *Penicillium expansum* made no growth on apples at 10° after two weeks, but the former grew on corn-meal agar at 0° from the beginning of the experiment and the latter had made a start at that temperature by the end of the fourth day. *Volutella fructi* had made no

evident start on apples at 5° after two weeks, but grew on corn-meal agar at 0° from the first. *Sphaeropsis malorum*, however, showed the same slowness in starting on corn-meal agar at low temperatures that it had

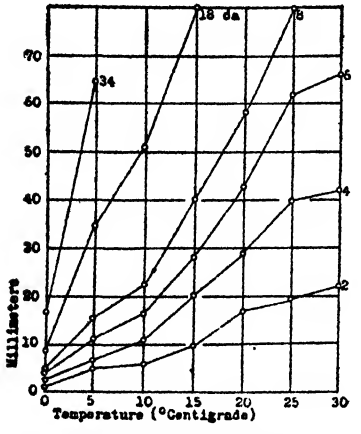


FIG. 14.—Graph showing the growth of *Alternaria* sp. on corn-meal agar in petri dishes.

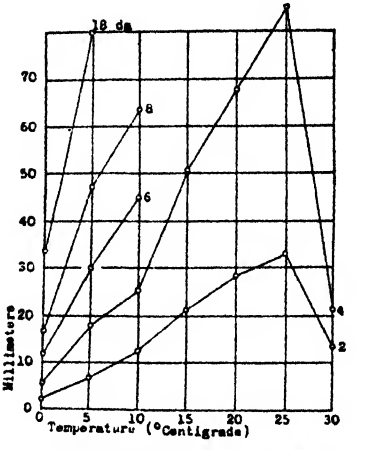


FIG. 15.—Graph showing the growth of *Botrytis cinerea* on corn-meal agar in petri dishes.

on apples and, contrary to the fungi already mentioned, it grew at a lower temperature on apples than on the agar. The above contrasts show that with nearly all of the fungi studied the period of incubation

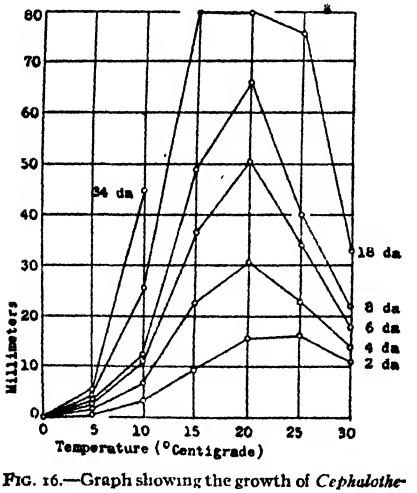


FIG. 16.—Graph showing the growth of *Cephalothecium roseum* on corn-meal agar in petri dishes.

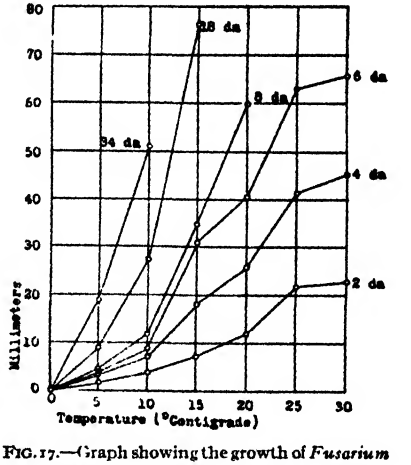


FIG. 17.—Graph showing the growth of *Fusarium radicicola* on corn-meal agar in petri dishes.

that was pointed out as important in the experiments with apples has much less significance when the same fungi are grown on corn-meal agar.

The contrast between the growth on apples and that on agar at low temperatures becomes still more striking when we consider that in the

plotting of the curves for figures 14 to 23 and also for figure 25 the growth during the 24 hours at laboratory temperature was always deducted from the final growth, a thing which could not be done in the case of the

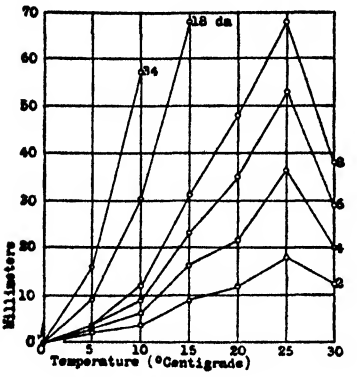


FIG. 18.—Graph showing the growth of *Glomerella cingulata* on corn-meal agar in petri dishes.

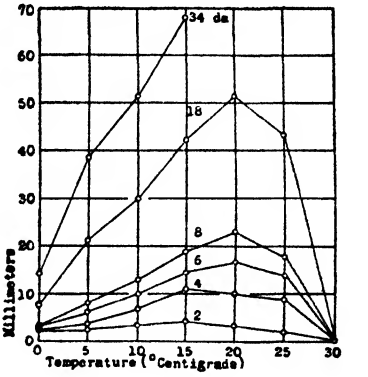


FIG. 19.—Graph showing the growth of *Neofabraea malicorticis* on corn-meal agar in petri dishes.

apple experiments, as the effects of the fungi were not evident at the end of one day.

It was thought that, since the agar probably remained practically uniform at the different temperatures and the apples were evidently

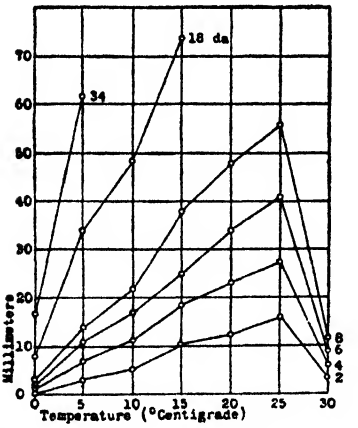


FIG. 20.—Graph showing the growth of *Penicillium expansum* on corn-meal agar in petri dishes.

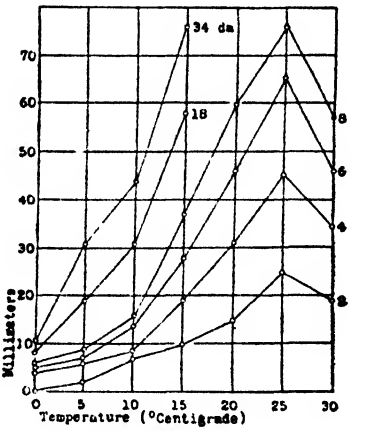


FIG. 21.—Graph showing the growth of *Sclerotinia cinerea* on corn-meal agar in petri dishes.

ripened and weakened by the higher ones, the optimum for some of the fungi might be lower on the former than on the latter and that there might be more sudden drops above the optimum with the former. A comparative study of the results, however, does not indicate this.

Some of the fungi that furnished little temperature contrast on the apples gave quite striking ones on agar. This is especially noticeable with *Cephalothecium roseum*, but it also occurs with *Alternaria* sp. and *Botrytis cinera*. On the other hand, the species of Sclerotinia, Penicillium, and Sphaeropsis used have given relatively less temperature contrast on corn-meal agar than on apples. (See figs. 1, 2, 25.) A medium favorable to the fungus apparently intensifies the temperature contrasts.

While there are the differences already pointed out in the temperature responses of the various fungi on the two media, the curves plotted for growth on corn-meal agar are on the whole in quite close agreement with those for growth on the fruit, showing that the temperature response is largely determined by the organism itself.

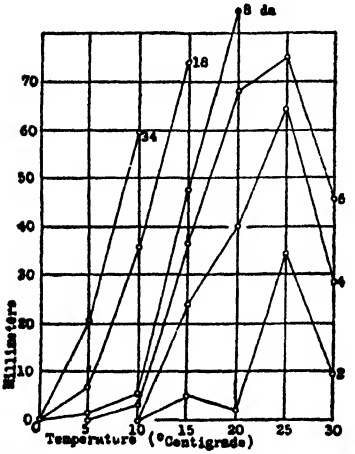


FIG. 22.—Graph showing the growth of *Sphaeropsis malorum* on corn-meal agar in petri dishes.

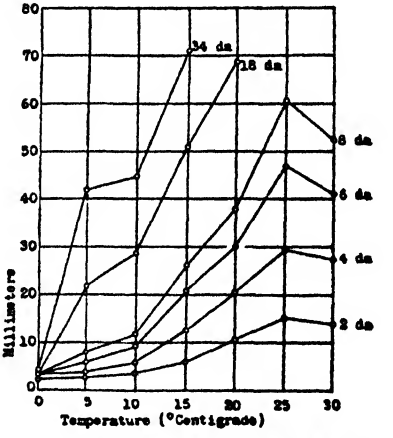


FIG. 23.—Graph showing the growth of *Volutella fructi* on corn-meal agar in petri dishes.

It will be noted that the curves for the petri-dish experiments, like those for the apple rots, have been plotted on the basis of the diameter rather than the area, thus giving relatively low values to the growth in the larger colonies and making the contrasts too small between the high and low temperatures and between the early and late stages of the experiment.

GERMINATION AT LOW TEMPERATURES

The above experiments with petri dishes furnish data as to the growth of the fungi at various temperatures when the cultures had been exposed for 24 hours in the laboratory before being placed at the temperatures of the experiment. A later test was made on corn-meal agar in which the petri dishes were inoculated as already described but were placed at the desired temperatures immediately. Notes were taken at the times indicated in Table IV.

TABLE IV.—*Results of germination experiments with various apple-rot fungi.*[a]

Fungus.	Germination at 10° C. after—		Germination at 5° C. after—		Germination at 0° C. after—	
	10 days.	31 days.	10 days.	31 days.	22 days.	31 days.
Alternaria sp.	+	+	+	+	−	+
Aspergillus niger	−	−	−	−	−	−
Botrytis cinerea	−	+	−	+	−	+
Cephalothecium roseum	−	+	−	+	−	−
Fusarium radicicola	−	+	−	+	−	−
Glomerella cingulata	+	+	+	+	−	−
Penicillium expansum	+	+	+	+	+	+
Pestalozzia funerea	−	+	−	+	−	−
Sclerotinia cinerea (apple)	−	+	−	+	−	+

[a] + indicates germination; − indicates failure to germinate.

There was little contrast between the germination at 10° and that at 5°. At both these temperatures *Alternaria* sp., *Glomerella cingulata*, and *Penicillium expansum* had germinated by the end of 10 days and all of the fungi but *Aspergillus niger* by the end of 31 days. At 0° none of the fungi but *P. expansum* had germinated at the end of 22 days and only the species of Alternaria, Botrytis, and Sclerotinia were added to this by the end of 31 days.

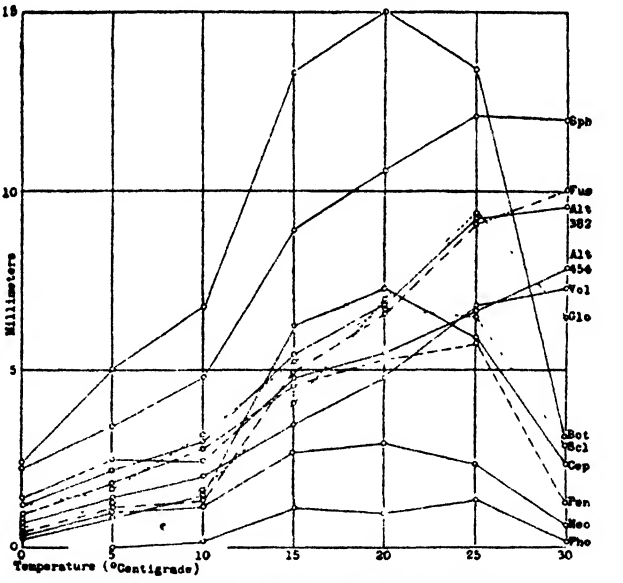


FIG. 24.—Graph showing the comparative growth of the different rot fungi on corn-meal agar petri dishes, first experiment. The curves show the average daily increase in diameter during the first six days of the experiment.

At 10° *Alternaria* sp., *Botrytis cinerea*, *Fusarium radicicola*, *Penicillium expansum*, and *Sclerotinia cinerea* were fruiting by the end of the month, and *Cephalothecium roseum* had made a good growth, but *Glomerella cingulata* and *Pestalozzia funerea* had produced colonies but a few millimeters in diameter. After 31 days at 5° the species of Alternaria, Botrytis, Penicillium, and Sclerotinia used were fruiting, while the colonies of Cephalothecium, Fusarium, Glomerella, and Pestalozzia had barely made a start. At 0° *P. expansum*

had produced colonies 3 mm. in diameter and was fruiting by the end of the month; *B. cinerea* and *S. cinerea* had produced colonies 0.5 mm. in diameter, but *Alternaria* sp. had little more than germinated.

A second series of germination tests was made in which the above results were confirmed. In this experiment it was also found that the

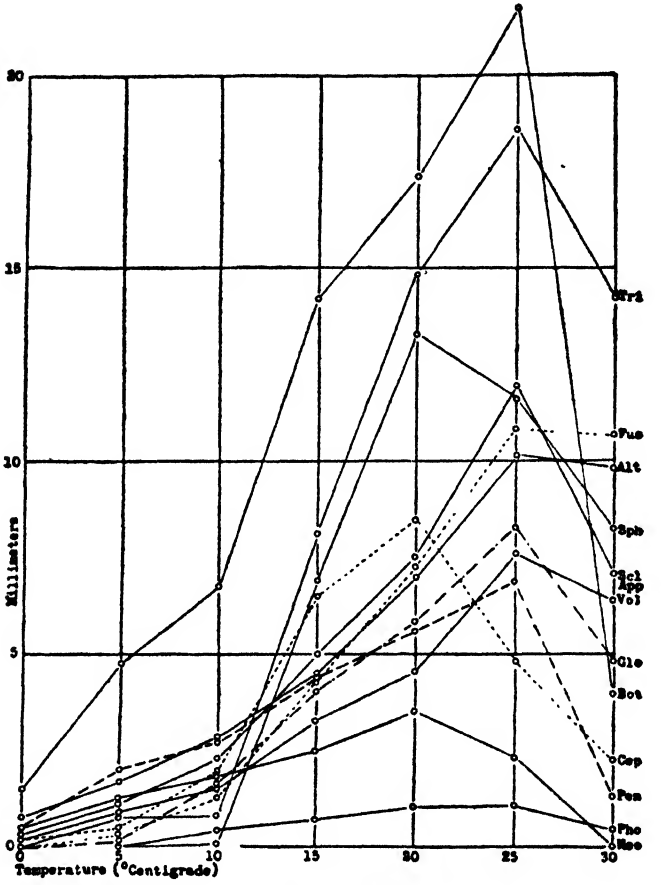


FIG. 25.—Graph showing the comparative growth of the different rot fungi on corn-meal agar petri dishes, second experiment. The curves show the average daily increase in diameter. With *Botrytis cinerea* and *Trichoderma* sp. they are based on the increase in diameter between the second and fifth days after inoculation, with *Sphaeropsis malorum* on the increase between the second and seventh days, and with all the other fungi on the increase between the second and eighth days.

spores of *Neofabraea malicorticis* germinated at 0° and that the fungus had fruited at that temperature at the end of 60 days.

The above results show that with a favorable medium *Penicillium expansum* is able to make a better start at 0° than any of the other fungi. The fact helps to explain its common occurrence in cold storage and is in agreement with the idea already suggested that its failure to grow on rather immature fruit at 0° is due in part to its weak parasitism.

DISCUSSION OF RESULTS

A comparison of the different tables and figures shows that the temperature response of a particular organism is modified by the medium upon which it is grown. The contrasts have been particularly striking between the behavior of the fungi on the living fruit and on dead organic matter. On corn-meal agar most of the fungi have grown from the first day even at rather low temperatures, while on apples there has usually been an initial incubation period of very slow growth which gradually passed over into a later one in which decay proceeded rapidly. In the initial stages of growth on the fruit the fungus must live almost entirely as a parasite and has not had time to build up reserve food material or secrete enzymes in quantity, while in the later stages it has a continually increasing mass of mycelium and the possibility of obtaining much of its food saprophytically from the tissue already broken down. The initial incubation stages of decay have been much more inhibited by low temperatures than the later stages, leading in some cases to a delayed development of rot and in others to a total prevention of it at a temperature at which the fungus was capable of making a rapid saprophytic growth. The inhibition at low temperatures was more pronounced with immature fruit than with mature specimens and with weak parasites than with strong ones.

Glomerella cingulata was unable to make any growth at 5° or 10° and *Fusarium radicicola* at 15° on Yellow Newtown or Winesap apples, but both fungi grew well on corn-meal agar at all three of these temperatures. *Neofabraea malicorticis* and *Penicillium expansum* had produced no evident rot on Winesap apples at 0°, 5°, or 10° at the end of two weeks, but they finally caused fruit decay at all these temperatures. Both grew from the first day at 5° and 10° when inoculated into corn-meal agar, and both had made a measurable growth on this medium at 0° by the end of the second day. Similar contrasts were found with several of the other fungi, but were not evident with *Sclerotinia cinerea* and *Sphaeropsis malorum*, two of the more distinctly parasitic fungi tested. The former was comparatively little delayed at low temperatures on either fruit or corn-meal agar; the latter was greatly delayed at 0°, 5°, and 10° on both food materials.

The early stages of the rots were not only partially inhibited by low temperatures but sometimes entirely prevented at temperatures at which the later stages could develop. In the commercial cold-storage experiments it was found that *Penicillium expansum* was able to make a good growth at 0° on fruit on which it was entirely unable to make a start at that temperature. This could not have been a question of germination, as the fruit was in the center of the barrel, and there would have been ample time for this to have taken place before the cooling was completed. This fact is very significant in showing the value of immediate as com-

pared with delayed storage. Most of the rot fungi are able to make but a very slow growth at 0° and 5° if placed at once at those temperatures; but if they go into storage with the accumulated energy of a week's growth or more the results are likely to be very different.

With the weakest parasites the maturity of the fruit has also had a modifying effect, the fungi having a lower minimum on the ripe than on the green fruit. This was evident with the apples of a particular variety inoculated at the same time and is also the most probable explanation for some of the contrasts in the results on the different varieties tested. At 0° *Penicillium expansum*, for instance, was able to produce a measurable rot on the York Imperial and Ben Davis by the end of 5 weeks, on the Yellow Newtown and Winesap that were slightly greener by the end of 8 weeks; but on still less mature York Imperial and Arkansas apples it had produced no rot at this temperature after 18 weeks.

Experiments have shown that practically all of the rot fungi are able to germinate at 5° and most of them at 0°. Germination is somewhat delayed at the latter temperature; but this does not seem to be the whole explanation for delayed growth on fruit at low temperatures, since in most of the experiments made time was given for germination before the fruit was placed in storage. It is of interest to note that while *P. expansum* was the first to germinate at 0° it is comparatively slow in making a start on fruit at that temperature. With *P. expansum* and probably with others of the weaker parasites cold storage inhibits the initial stages of the rotting more than it does germination.

While the minimum temperature for the various fungi has varied greatly with the food material, there has been little shifting of the optimum under the same conditions. *Alternaria* sp. and *Fusarium radicicola* have an optimum at 30° or above; *Botrytis cinerea, Glomerella cingulata, Penicillium expansum, Sclerotinia cinerea, Sphaeropsis malorum* and *Volutella fructi* at 25°, and *Cephalothecium roseum* and *Neofabraea malicorticis* at 20°.

The temperature curves are all plotted on the basis of diameter of the rot or of agar colony. On this basis the increased growth with a rise of temperature usually comes within the limits of the Van't Hoff law, the diameter becoming two to three times greater with each 10° rise in temperature; but as has already been pointed out, this method of estimation does not give full value to the increase in size in the larger rots and larger agar colonies. Curves plotted on the basis of area would come within the limits of the Van't Hoff law in most cases; but there would be some striking exceptions, *Sphaeropsis malorum* producing a rot area 16 times as great at 25° as at 15°, and *Botrytis cinerea* an area 25 times as great at 20° as at 10°.

SUMMARY AND CONCLUSION

(1) The behavior of *Alternaria* sp., *Botrytis cinerea, Cephalothecium roseum, Fusarium radicicola, Glomerella cingulata, Neofabraea malicorticis, Penicillium expansum, Sclerotinia cinerea, Sphaeropsis malorum*, and

Volutella fructi has been studied at different temperatures and under various conditions.

(2) In the inoculations on apples all of the fungi grew at 0° except the species of Fusarium and Glomerella, the former making no growth at 15° and the latter none at 10°. *Sphaeropsis malorum* had produced no evident rot at 15° by the end of one week nor the species of Penicillium and Neofabraea at 10° by the end of two weeks, while *Sclerotinia cinerea* produced measurable rots at 5° in one week and at 0° in two weeks. *Neofabraea malicorticis* had an optimum at 20°, *Fusarium radicola* at 30°, and all the other fungi at 25°. With most of the organisms the growth rate dropped off rapidly above 25°, but with the exception of *N. malicorticis* all made some growth at 30°.

In commercial cold-storage experiments on rather immature fruit *Penicillium expansum* continued to develop at 0° in the case of delayed storage, but was unable to grow at that temperature in the case of immediate storage.

(3) On corn-meal agar in petri dishes all the fungi used but the species of Cephalothecium, Fusarium, Glomerella, and Sphaeropsis grew at 0°, while these four made a fair growth at 5°. The optimum and maximum temperatures for the various fungi were the same as in the fruit-inoculation experiments.

(4) The spores of the species of Alternaria, Botrytis, Penicillium, and Sclerotinia had germinated in corn-meal agar at 0° at the end of one month; but those of the species of Aspergillus, Cephalothecium, Fusarium, Glomerella, and Pestalozzia had not. At 5° germination had taken place with all of them but *Aspergillus niger* at the end of the month.

(5) The temperature responses of the various fungi have been greatly modified by the food material upon which they were grown. *Fusarium radicicola* and *Glomerella cingulata* had a lower minimum temperature on corn-meal agar than on fruit and the early growth of the species of Alternaria, Botrytis, Neofabraea, and Penicillium used was much less inhibited on corn-meal agar at low temperatures than on the apples. In the case of fruit inoculations with *Penicillium expansum* and others of the weaker parasites the minimum has varied with the maturity of the fruit.

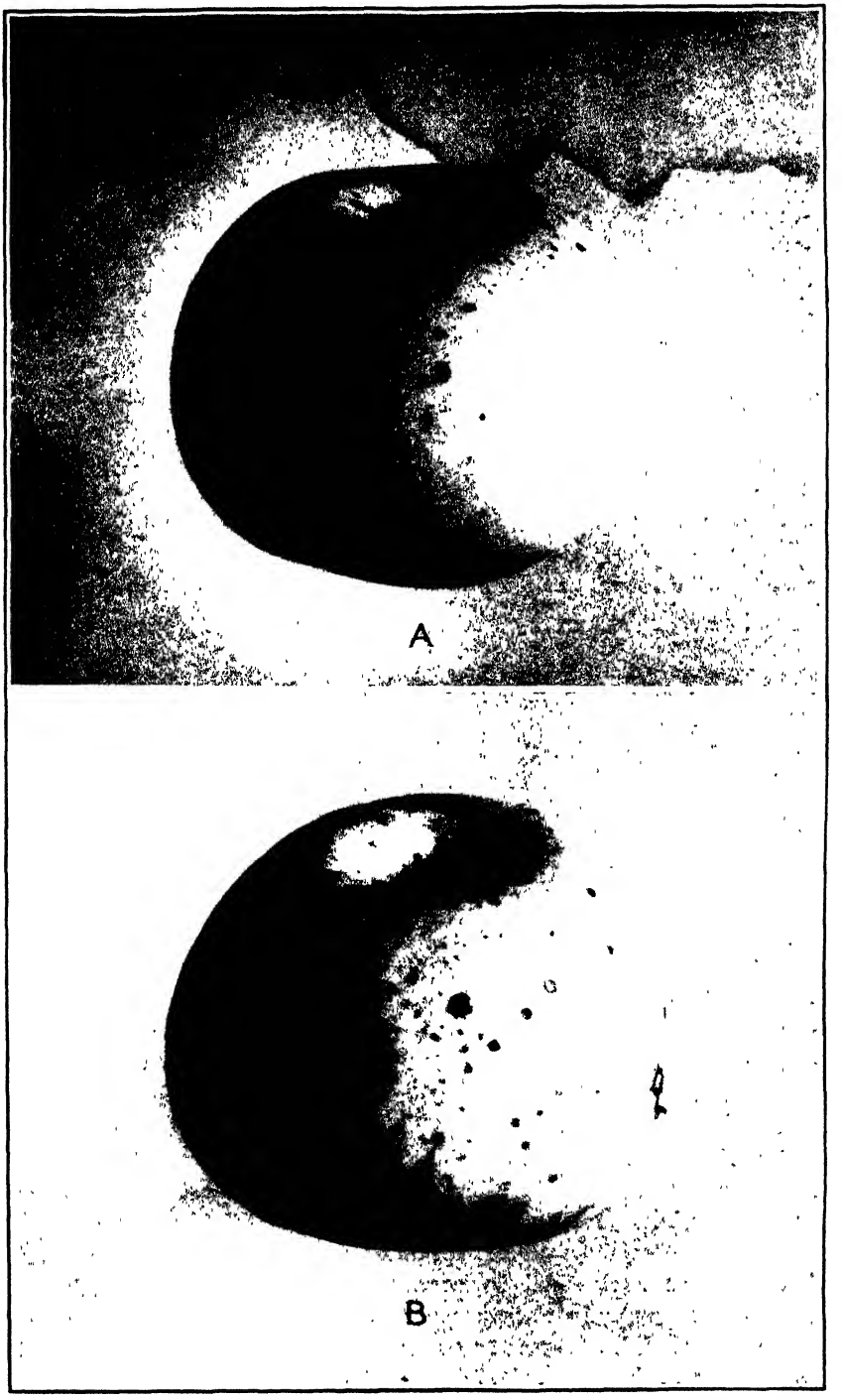
(6) With most of the fungi the initial incubation stages of growth on the fruit have been more inhibited by low temperatures than the later ones. With the weaker parasites like *Penicillium expansum* it is apparently this initial stage of decay rather than germination that determines the minimum temperature for fruit rot.

(7) The results show the importance of immediate as compared with delayed storage; the value of temperatures of 5° or 10° in short periods of storage and of 0° in longer ones and that the minimum temperature will vary with the prevalent fungus and with the variety and maturity of the fruit.

PLATE 1

A.—Grimes Golden apple affected with blackrot, showing character of the natural infections with *Sphaeropsis malorum* that were used in the temperature experiments.

B.—Ben Davis apple affected with bitter-rot, showing the character of the natural infections with *Glomerella cingulata* that were used in the temperature experiments.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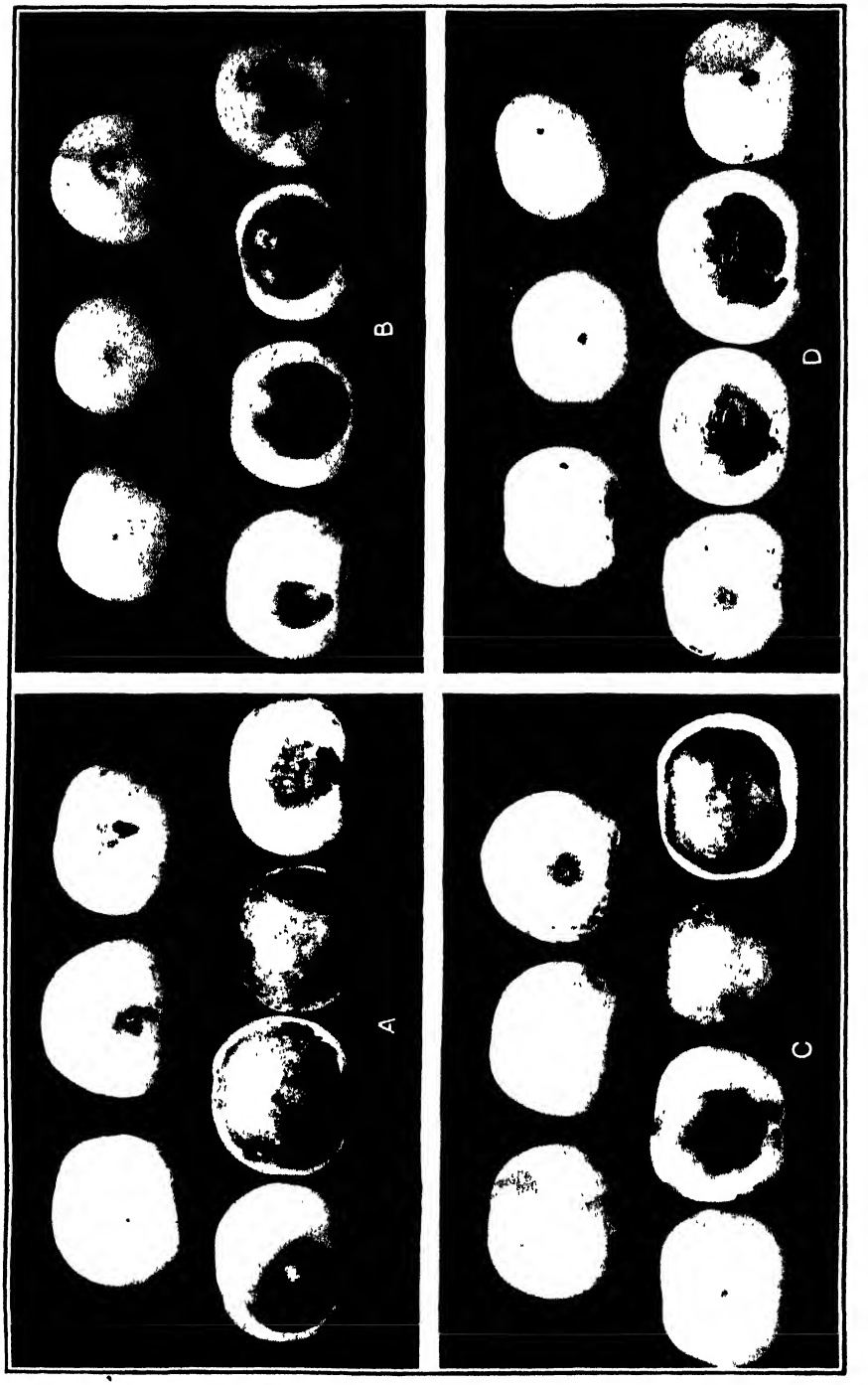
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PLATE 2

Yellow Newtown apples, showing the behavior of *Sclerotinia cinerea* at different temperatures, the apples being placed in the order of the temperatures used:

A.—Results obtained with a culture of *S. cinerea* from a peach after two weeks at 0°, 5°, 10°, 15°, 20°, 25°, 30° C.

B.—Results obtained with a culture of *S. cinerea* from an apple after one week at 0°, 5°, 10°, 15°, 20°, 25°, 30° C.

C.—Results obtained with a culture of *Sphaeropsis malorum* after two weeks at 0°, 5°, 15°, 10°, 20°, 25°, 30° C.

D.—Results obtained with a culture of *Penicillium expansum* after two weeks at 0°, 5°, 10°, 15°, 20°, 25°, 30° C.

PLATE 3

Yellow Newtown apples, showing the behavior of apple-rot organisms at different temperatures, the apples being placed in the order of the temperatures used.

A.—Results obtained with a culture of *Volutella fructi* after two weeks at 0°, 5°, 10°, 15°, 20°, 250, 30° C.

B.—Results obtained with a culture of *Glomerella cingulata* after two weeks at 0°, 5°, 10°, 15°, 20°, 25°, 30°, C.

C.—Results obtained with a culture of *Neofabraea malicorticis* after three weeks at 0°, 5°, 10°, 15°, 20°, 25°, 30° C.

D.—Results obtained with a culture of *Neofabraea malicorticis* after five weeks at 0°, 5°, 10°, 15°, C.

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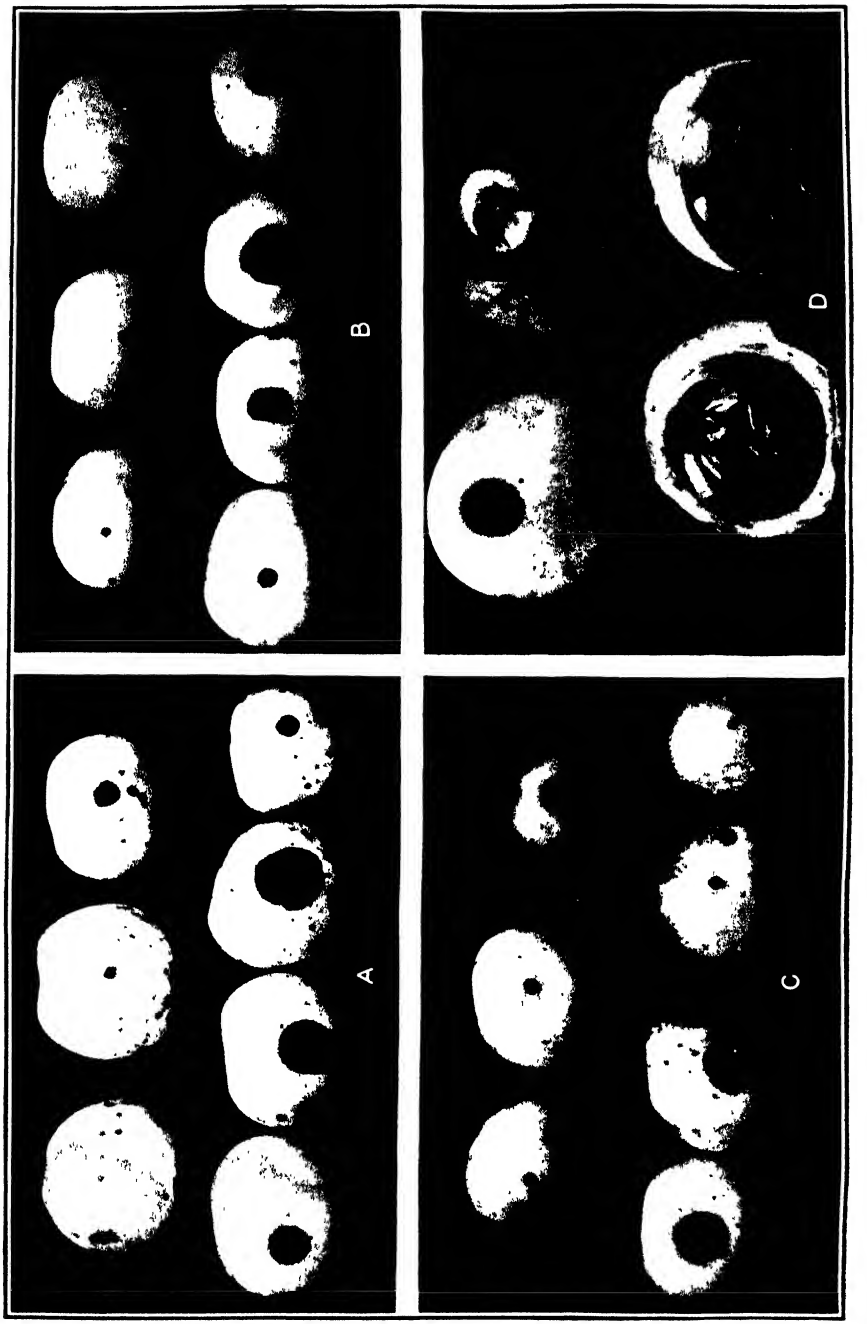
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MECHANISM OF TUMOR GROWTH IN CROWNGALL

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The ultimate cause of cell proliferation in crowngall is the Schizomycete *Bacterium tumefaciens* Sm. and T., as set forth elsewhere (1, 2, 3);[1] but the mere mechanical irritation due to the presence of a few rod-shaped bacteria in the tissues can not be the direct cause of the proliferation, since other species of bacteria either have no specific action when inoculated into plants or some quite different action, such as a wilt of the foliage following an excessive multiplication of bacteria in the vascular system, as, for example, in the disease of cucurbits due to *Bacillus tracheiphilus*, or a soft rot of shoots and tubers following an excessive multiplication of bacteria in the intercellular spaces with solvent action on the middle layers of the cell wall, as, for example, in the potato rot due to *Bacillus phytophthorus*. In each of these types of plant disease (tumor, wilt, and softrot), the ultimate cause is a bacterial infection; but the immediate or proximate cause of the phenomena, except perhaps in case of vascular embolisms, must be the chemical or physical action of enzyms or other substances produced or activated by the bacteria as a result of their metabolism, with a corresponding *reaction* on the part of the plant.

For a long time I have tried to think out the rationale of what goes on in the cell following the introduction of the crowngall organism. What we see is excessive and abnormal multiplication of the tissues resulting in a tumor, a hyperplasia. What we would wish to know is the mechanism of the growth—that is, the chemical or physical stimulus behind the observed phenomena—since, if we can comprehend it in the plant, we may be able to apply our knowledge to the understanding of similar phenomena in man and animals.

In this paper, therefore, I have ventured to offer some suggestions (supported by experiments) as to the proximate cause of the abnormal

[1] Reference is made by number to "Literature cited," pp. 185–187.

cell proliferation in crowngall—a proliferation in opposition to the needs and well being of the plant and one following the general law of development of animal neoplasms, as I have shown elsewhere (4,18). It is believed that the experiments here detailed have important bearings not only on the origin of crowngall and of other tumors in plants, including those due to nematodes and to gall flies, but also on the origin of various animal tumors. For the most part, however, I shall here leave the reader to draw his own conclusions respecting remoter applications.

At first I conceived of the crowngall phenomena as much more complex than it really is. I thought of endotoxins and other complex substances of unknown chemical structure as probably the cause of the excessive cell proliferation, not, however, entirely excluding simpler substances, such as ammonia compounds.[1] Absorbed in other features of the investigation, I limited myself for a long time to turning over in my mind various phases of the problem with what help I could obtain from literature, but in June of last year (1916), having reached, theoretically, what seemed to me to be dependable and necessary conclusions, I began to make simple experiments, some of which have proved of great interest, and the more important of which are here first described, though I called attention to them briefly in October (5, 6).

In the beginning I had what I now believe to be a wrong conception of growth. I looked upon it as something that an outside substance could directly stimulate into development, but probably it is not that (Weigert, Ribbert, Loeb). Growth is the normal function of cells. They are always multiplying when they are not inhibited by one thing or another. Growth, then, if this view is correct, comes about not by the direct application of stimuli, but indirectly *by the removal of various inhibitions.* Under normal conditions the physiological brakes are on at all times, more or less, in both plants and animals, and only when they are entirely or largely removed in particular areas do we observe an unlimited cell proliferation resulting in the hasty and peculiar growths known as neoplasms or cancers. What, then, removes the normal growth inhibitions? And what are these inhibitions? Cold is one source of inhibition, insufficient oxygen and drouth are other causes of inhibition, but we have to do with none of these inhibitions or their opposites, nor with any others in so far as they act on the plant as a whole. The inhibition remover we are in search of is *one that acts locally, disturbing tissue equilibriums within limited areas.*

When all other conditions are favorable, such as abundant food supply, water supply, air supply, and right temperature, cell multiplication may still be held in check—that is, in unfertilized eggs—as Jacques Loeb has

[1] In a previous paper (1, p. 175), ammonium acetate is especially mentioned as a possible cause of tumor stimulus.

shown, by the surface tension of the cells. This is due, he thinks, to lipoids or fatty bodies present in their surface membrane. If one changes the surface tension of egg cells by the introduction of alkalies or fatty acids or even by punctures, growth takes place in the absence of fertilization, and a new animal (the fatherless frog or sea urchin) results. This is the problem of starting the normal physiological growth of a whole organism from an egg in the absence of sexual impact and it has been solved by withdrawing water from the surface of the egg cell. This has been done in some species by pricking the eggs, and in others by increasing the osmotic pressure of a surrounding fluid. In malignant tumors the problem is one of explaining a continuous and excessive *local growth*, abnormal in that it is outside of physiological control, but it is, I believe, a problem to be solved in the same way, viz, by showing that the overgrowth is due to a removal of the local growth inhibitions by local increase of osmotic pressures. As I now see it, tumor growth is not at all a chemical phenomenon, but rather a purely physical one, a matter of varying molecular vibrations, a development dependent on locally increased osmotic pressures which cause a movement of water and foodstuffs into the affected area. In crowngalls the removal of growth inhibitions is brought about, I think, by the physical action of substances liberated within the tumor cells as the result of the metabolism of the imprisoned bacteria. Occasionally in this paper I have used the convenient word "stimulus" in an unqualified way, but by it I mean always and only *the remover of an inhibition.*

If the cell proliferation in crowngall is due to substances liberated within the cell by the parasite, as it seems reasonable to suppose, they must be substances either identical with or at least not differing greatly in their physical or physiological action from those acting on the non-parasitized cell during normal growth and division. Of this there can be little doubt for several reasons and especially for the reason that there is no evidence of chemical injury either in the tissues surrounding a crowngall or in the tumor cells themselves, since they grow and multiply with a rapidity only to be compared with that of the cells of normal young tissues. This at once removes from consideration all actively poisonous (killing) substances and greatly simplifies the problem.

Various weak (dilute) poisons are known to cause cell proliferations in plants—that is, copper salts (7, 8), vaseline and paraffin oil (9),[1] salts of

[1] Schilling's interesting paper (9) was received at the Department of Agriculture in November, 1915, but I did not know of its existence until October of the following year—i. e., after my own experiments were completed, except for a few repetitions and some tests with poisons in great dilution. His results (numerous large-celled intumescences) were obtained by vaselining or paraffining the surface of twigs of various species: Aesculus, Sambucus, Syringa, Artocarpus, Philodendron, Ribes, Spirea, etc. Since this note was in type I have discovered that Schilling's work is only a repetition and extension of work published in 1910 by the Russian botanist, P. Wisniewski (22).

lithium (10, 11),[1] and the excretions of the larvæ of gall flies, of certain nematodes, and of various fungi. Other substances are known to cause cell proliferations in animals—that is, Sudan III and scarlet-red (12), repeated applications of tar (13), excretions of Bilharzia, etc. As bearing on the origin of neoplasms, these facts are extremely suggestive; but for obvious reasons chemicals which can not be supposed to occur in genuine neoplasms as the result of their own metabolism and can not be assumed to be excreted into them by parasites either actually or presumptively present, need not be considered here, however effective they may be as a cause of cell proliferation. In our search for the direct exciting cause of tumors we need consider only the excretions of known tumor-producing organisms; but since little or nothing is known concerning the growth-exciting substance, or substances, produced in animal neoplasms, or liberated in plants by the various gall-forming fungi, or by gall flies and gall nematodes, we may for the present confine our attention exclusively to the bacterial tumors of plants. This narrows down the problem to a few species of bacteria and even some of these—that is, *Bacterium savastanoi* (the cause of olive tubercle) and *Bact. beticola* (the cause of beet tubercle)—are negligible so far as the purposes of this paper are concerned, because I regard the tumors they produce as granulomas, not neoplasms. In fact, thus far in seeking for the physical-chemical origin of neoplasms I have sought only for answers to the following questions:

(1) What are the products of the bacterial metabolism of *Bact. tumefaciens*, the crowngall organism?

(2) Are any of these products capable of inducing cell proliferation when injected into the growing plant?

(3) If so, is this substance a sufficiently common product of other bacterial and nonbacterial tumor-producing parasites so that it may be regarded hypothetically as produced by all of them and consequently as the exciting cause of all bacterial, fungus, nematode, cynipid, and other vegetable galls, or must we suppose that various chemical growth excitants exist?

(4) And finally to what extent can these facts be supposed to apply to human and animal neoplasms?

In case of the crowngall organism I had to begin with the simpler substances, because I have as yet no knowledge of the more complex products of its metabolism, if any such exist, and certainly no very poisonous substances are produced in the crowngall, any more than in animal

[1] In this connection should be mentioned especially Caroline Rumbold's recently published results of chestnut-bark injections (11). With substances injected in the hope of controlling the Endothia chestnut-bark disease, and especially with lithium carbonate, she succeeded in causing numerous conspicuous islands of xylem to develop in the middle of the phloem. These caused bulges in the bark, visible externally. I have had the pleasure of examining her sections, and verifying her statements. The islands are developed from a cambium, the growth being always from the outer face of a bundle of bast fibers toward the surface of the stem. I could not satisfy myself as to the origin of this cambium from the normal cambium of the stem (Solereder), but it is plain that the occurrence of these islands of xylem in the phloem (they appear to be that rather than islands of phloem in xylem) is correlated with a scanty production of the normal wood of the season.

neoplasms (until secondary infections occur), because the tumor cells are not visibly injured except that they stain somewhat differently from normal embryonic tissues, are unduly excited and, along with greatly increased vegetative tendencies, have lost the whole or a great part of their polarity (orderly arrangement of cells) and also most of their power to differentiate tissues. The most conspicuous results of the growth of *Bact. tumefaciens* in culture media containing grape sugar and Witte's peptone are ammonia and alcohol (Alsberg, Brewster, Woodward) and an acid, said by Dr. Carl L. Alsberg, Chief of the Bureau of Chemistry, to be acetic acid in case of the Daisy strain with traces of oil or of a fatty acid, and by Dr. J. F. Brewster, also of the Bureau of Chemistry, one of the fatty acids (but not acetic acid) in case of the hop strain. These substances are produced by the bacterium in flasks of Jena glass containing only calcium carbonate, water, grape sugar, and peptone, and undoubtedly they are also produced by it within the cells of the tumor. According to Dr. H. E. Woodward, of the Bureau of Chemistry, small quantities of amins (but not trimethylamin) are produced by the hop strain—that is, primary or secondary amins.[1] Probably the organism also

[1] Since these two sets of analyses were made Dr. Alsberg has kindly had further analyses made for me of *Bact. tumefaciens* plated from Flats poplar, from Massachusetts rose, and from another Paris daisy gall. These were flask cultures grown over calcium carbonate in distilled water containing 1 per cent Witte's peptonum siccum, and 1 per cent Merck's c p. dextrose, sterilized by discontinuous steam heat on 4 consecutive days and allowed to stand some days before inoculation. These analyses, which were made by Drs. Brewster and Woodward, at the end of 8 to 9 weeks, show, as in the case of the previous analyses of the hop strain, that alcohol, ammonia, and amins are formed and small quantities of fatty acids. Formic acid was detected in each of the nine flasks No acetic acid was found in flasks inoculated with the daisy strain, but it occurred in those inoculated from poplar and rose. Aldehyde and acetone were also detected in each flask. No trimethylamin was found. The analyses of a second series of the hop strain were made in January on cultures somewhat older than the first series. (See page 186.)

In each instance I personally inoculated the flasks with great care, using agar subcultures made originally from single poured-plate colonies, flaming the necks of the flasks thoroughly in advance, turning them down on their sides to inoculate and keeping them open for a moment only in still air in a clean culture chamber, but even so there are always slight chances of contamination To further eliminate these chances several flasks were used for each strain, several were held uninoculated for control, and the others were watched to see that growth came on normally for this organism. Finally agar poured-plates were made from each inoculated flask to judge of its continued purity and subcultures from each were tested out on plants and found to be infectious. The flasks, including those which were not inoculated and all of which had remained sterile, were then turned over to the chemists, who analyzed each one separately, with concordant results except as indicated below.

CHEMISTS' REPORT OF ANALYSES OF FLASK CULTURES

"Three flasks each of cultures marked Flats Poplar, Rose, and Resistant Daisy, dated October 21, 1916, were received from Dr. Smith on December 16, 1916. With these were also three control flasks which had not been inoculated

"The flasks were opened and each worked through in order until finished, beginning December 18, the methods being as follows

"The contents were filtered off from undissolved calcium carbonate. A small portion of the filtrate acidified with acetic acid and treated with a few drops of ammonium oxalate solution gave an abundant precipitate of Ca-oxalate showing that much calcium carbonate had been dissolved. This is true of all the flasks which had been inoculated. Those not inoculated gave no test for dissolved Ca. The remainder of the cloudy filtrate was distilled over milk of lime. Some of the distillations were done with the free flame, others at diminished pressure to determine whether the same products could be found at the lower distilling temperature (40°–50° C). In either case the products were the same.

"Small portions of the alkaline distillate were tested for alcohol, acetone, and aldehyd, and the main portion, after neutralizing, was given to Dr. Woodward to test for volatile alkali.

"Acetone was first indicated by the formation of iodoform at the ordinary temperature when 2 drops of 10 per cent potassium hydroxid solution and sufficient iodine solution to produce a faint yellow color

liberates a small amount of carbon dioxid (4, p. 21–22). These substances, therefore, must be the first things tested out experimentally on plants subject to crowngall. On the start it seemed to me probable that ammonia, given off in small quantities within the cell by the multiplying bacteria, must be the sole determining factor in the abnormal cell proliferation, since as a diffusible stimulant it would enter cells readily, would increase the osmotic pressure, and would tend to enter into soapy combinations with the lipoids of the cell surface, thus changing the surface tension beyond the point of physiological cell restraint, whereupon cell division would take place. At the same time that ammonia and other concentrated bacterial products move outward, toward the surface of the tumor, where usually growth is most abundant, water and dissolved foods would move inward into the tumor, thus supplying copiously, especially at the periphery of the tumor, both the necessary stimulus and the substances needed for the constantly increasing abnormal growth. Minute continuous doses of other alkalies might act on the cells in the same way, but, as already intimated, it is here necessary to consider only such substances as are likely to be liberated within the cells of the tumor by the metabolism of the crowngall organism. If the substance which removes the growth inhibition is ammonia or any other compound produced by the crowngall bacterium as the result of its growth within the cell then the rate of cell division in the tumor would depend on the rate of bacterial multiplication and metabolism within the cells and on the relative juiciness of the tissues, which together would determine the rate of osmotic movement, while the rate of the bacterial multiplication

---

were added to the distillate. That acetone was present was confirmed by adding to a portion of the distillate an equal portion of strong potassium hydroxid solution and then a few drops of 10 per cent alcoholic solution of salicyl aldehyd. An orange red color is developed on warming to 40°–50° C. (Csonka, Jour. Biol. Chem. 1916, 27, p. 209).

"The acetone was removed from a separate portion of the distillate by aerating a few minutes at 40° C., and when there was no further positive test for acetone, alcohol was detected by means of the KOH-iodine reaction when iodoform was produced abundantly on warming to 60° C.

"The presence of aldehyd was detected by the Tollens's silver reduction and the fuchsin tests.

"Acetone, alcohol, and aldehyd in varying amounts were found in the distillates from each of the nine inoculated flasks. None of these was found in the controls.

"The residue in the distilling flask, representing about one-half the original volume, was diluted to original volume with distilled water, 25 cc 5 *N* sulphuric acid were added and the mixture submitted to steam distillation. The distillate was neutralized with barium hydroxid solution, evaporated in vacuum to small volume, and the barium salts obtained by further concentration on the steam bath. There appeared to be a mixture of barium salts present, but as the quantities were small only qualitative tests were made. Acetic and small amounts of formic acids were detected in flasks A, B, C (Flats Poplar), and E, F, G (Rose). Flasks I, K, and L (Daisy) gave no indication of acetic acid; formic acid only was detected (silver nitrate reduction).

"No nonvolatile acids were found in the acid residue on extracting with ether and evaporating. No fixed acids were found on acidifying the calcium carbonate residue in the culture flask and extracting with ether.

"(Signed) J. F. BREWSTER.

"JANUARY 5, 1917."

REPORT ON VOLATILE ALKALI

"In the alkaline distillates from flasks A, B, C, E, and F the following tests were made:

Ammonia	Present.
Amines	Do.
Trimethylamine	Absent.

"(Signed) H. E. WOODWARD."

would depend on a variety of external conditions and of interrelations and interreactions between the host and the parasite, such as darkness, sunlight, heat, cold, presence or absence of oxygen, abundant or scanty food supply and water supply, inherited or acquired host resistance, varying normal alkalinity or acidity of the tissues, inhibiting action on the bacteria of the acid by-products of bacterial growth, etc., ample to explain all the observed variations in the rate of growth of crowngalls and of all other neoplasms. If, furthermore, we take into account the various kinds and degrees of reaction on the part of the host, as we must, then this hypothesis is ample to explain also the whole tribe of benign tumors and all receding malignant growths.

It seemed to me, therefore, that ammonia or some salt of ammonia must be the determining factor in tumor growth. If so, then by injecting dilute ammonia water into growing plants one ought to obtain, not typical tumors, of course, for that involves the idea of a weak continuing action, hard to bring about experimentally, but an active proliferation of cells for a short time, corresponding to the brief action of the injected substance. Some killing of tissues should be expected at the points injected, especially from action of the stronger solutions but at a distance where there would be feebler action, and generally with weak solutions there should be no wounding or killing effect, but only a very marked cell proliferation. I reached this point in my reasoning before I made any experiments. Some things I overlooked, as will be seen later.

I was supported in these conclusions by the following facts out of literature. As a boy I read in some boys' wonder book that coffee berries, said to be proverbially slow to germinate, might be germinated overnight by throwing them into strong ammonia water. I bought some ammonia with my first pocket money, tried it, and found it to be as stated, but I held no checks. That was 50 years ago. Recently I have repeated the experiment with the same results. I have extended the experiment also to date seeds and to some others known to be hard to germinate—that is, black-locust seeds, but only with doubtful results, and even in the case of coffee seeds some germinate promptly in distilled water, so that, curiously enough, the experiment which more than any other determined the trend of these researches would have had no influence on my thinking had it been made in the first place properly—that is, with a sufficient number of controls in pure water.

Furthermore, in 1903–1905 (7, 8), Dr. Hermann von Schrenk, of the Missouri Botanical Garden, observed numerous warty proliferations on the leaves of cauliflowers which were attacked by *Peronospora parasitica* and had been sprayed with the fungicide copper ammonium carbonate, and investigated their origin with the following results:

In his experiments Dr. von Schrenk readily obtained numerous warty growths on cauliflower leaves within five days' time by spraying upon them copper ammonium carbonate. He also obtained warts by the

application of minute doses of other copper salts free from ammonia. Also in a first series of experiments using 2 per cent ammonia water (0.837 per cent of actual ammonia) and 5 per cent ammonium carbonate he obtained warts, but, as these solutions had been applied by means of an atomizer which had previously contained the copper solutions, the oligodynamic effects of copper were suspected and the experiments with the ammonia and ammonium carbonate were repeated, using brushes so as to exclude copper contamination, but then with more or less contradictory results, so that the author was in doubt as to the action of ammonia, and concludes the paragraph as follows:

> While several of the sprayed spots undoubtedly formed intumescences as a result of spraying with ammonium carbonate or ammonia, owing to the negative results of several plants it will not be possible without further tests to definitely ascribe the formation of these intumescences to the ammonia.

For this reason also, no doubt, he does not mention ammonia as a cause of intumescences in his general summary, which I quote:

> The results of the present investigation may be briefly stated as follows:
>
> 1. Cauliflower plants sprayed with copper ammonium carbonate produced large numbers of intumescences as a direct result of the spraying.
>
> 2. Similar intumescences were produced by means of weak solutions of copper chloride, copper acetate, copper nitrate, and copper sulphate when sprayed in very fine drops on the surface of the leaves.
>
> 3. The intumescences were formed in larger numbers on the lower surface of the leaves than on the upper surface of the leaves.
>
> 4. Intumescences were formed independent of soil or atmospheric conditions, so that the heat and water supply had nothing to do with their formation [1]
>
> 5. Intumescences must be regarded as the result of the stimulating activity of chemical poisons, sprayed upon the leaf in weak solutions.
>
> 6. The stimulating activity exerted is probably due to the formation of compounds within the cells of high osmotic tensions, these compounds being either compounds formed by the copper salts with parts of the protoplast, or compounds formed as a result of a stimulus exerted, as evidenced by the presence of large amounts of oxidizing enzymes [2] [2 per cent gum guaiac test] as a result of an indirect stimulus exerted by the salts sprayed upon the leaf surface.

Rosen (14), who repeated Von Schrenk's copper-ammonium-carbonate experiments on cauliflower and extended them to cabbage with positive results on both plants, speaks only of the copper stimulus and apparently did not suspect the ammonia, but to me the presence of that very diffusible compound in suitable dilution explains in conjunction perhaps with carbon dioxid by far the larger part of the observed action.

To test out my conclusions respecting ammonia as the probable tumor stimulus, I first injected, in a preliminary way, small quantities of strong ammonia water (aqua ammonia, sp. gr. 0.90, 1 part, and distilled water 10 parts) into the young internodes of large castor-oil plants (*Ricinus com-*

[1] Excessive water supply and excessive heat are believed to cause intumescences on grape leaves. See Von Schrenk's papers (7, 8).

[2] Crowngalls also are very rich in oxidizing enzyms (1, p. 173).

munis). This strong-growing plant develops crowngall quickly when inoculated with *Bact. tumefaciens* (the hop strain) and is also well adapted to the experiments here described not only because the internodes are hollow and will hold a considerable quantity of fluid, but also because in the younger internodes, at least, the cavity is lined by living pith cells capable of astonishing proliferation when properly stimulated. The older internodes were also injected, but these generally yielded no results other than death of cells immediately in contact with the strong alkali. In the younger internodes, on the contrary, striking cell proliferations into the pith cavity were obtained repeatedly. That part of the pith which received the brunt of the alkaline action was in every case killed for the solution was much too strong; but the remoter tissues, especially the inner face of certain xylem bundles, proliferated freely, almost furiously, often for long distances, forming raised snow-white cushions of delicate cells, which in their morphology strongly suggested those cells which are to be seen growing from the margins of gum pockets, but were much more abundant and active (Pl. 4 and 5). The cell proliferations, which in this case arise from the tissues of the vascular bundle or its sheath (inner face of the xylem or of the endocycle), are apparently much more abundant than one would expect for the repair of a simple wound. These cushions have the appearance of tissues growing rapidly under an active stimulus of some sort both because of the volume of the growths visible within a given short time and because of the great number of the cells composing them.

When these results were obtained with the strong ammonia two questions arose:

(1) Whether the growths were simply unusually active wound proliferations—that is, exclusively repair cells, or were really in part proliferations due to the stimulating (inhibition-removing) action of the alkali, and not exclusively to its killing (wounding) action;

(2) Whether the same results could be obtained with the various salts of ammonia.

Respecting the first inquiry we might conceive the growth response to be due (1) to the killing effect of the ammonia, the proliferations being simply the development of wound-repair tissue, in which case one ought to be able to obtain the same results by use of many other injurious substances, and killing effects should always be visible, while greatly diluted ammonia would then have no visible effect; or (2) to a specific effect of the ammonia due to the removal of physiological (or physical) growth inhibitions, in which case it should be excessive as compared with ordinary wound-repair tissue, should be obtained with very weak aqua ammonia (high dilutions) in the absence of any wounds, and should not be obtained from the use of acids or other poisons unless they also have a specific effect on the cell membrane; or (3) a nonspecific, water-attractive

(osmosis-inducing) purely physical effect, in which case many other substances would cause it, even in great dilution, and there would be then, also, no evidence of any wounding of the tissues except in case of excessive doses.

These various inquiries formed the basis of further experiments.

First, I tested out in Ricinus stems, and in part also in young green tomato fruits the effect of the following compounds: Urea, ammonium carbonate, ammonium tartrate, ammonium citrate, ammonium malate, ammonium acetate, ammonium sulphate, ammonium sulphite, ammonium nitrate, ammonium lactate, ammonium chlorid, ammonium oxalate, ammonium formate, ammonium salicylate, ammonium succinate, dibasic ammonium phosphate, and monobasic ammonium phosphate, with generally positive results and often very striking ones, and also once in case of the last-mentioned substance with very unexpected structural transformations to be described later.

To return now to the individual action of the various salts of ammonia. I obtained in the pith cavity of Ricinus and in the interior of green tomato fruits striking proliferations with a variety of these salts, as may be seen by consulting the accompanying plates, and that too whether the tissues were or were not visibly injured. Some of these substances at first were used too strong and exerted a local killing effect, but remote from these wounds fine proliferations were obtained from the apparently uninjured surface layer of the pith in Ricinus stems and from the inner pericarp wall in the green tomato fruits. Special attention is called to the outgrowths on the uninjured inner face of the green carpels of the tomato as a result of the action of ammonium carbonate (Pl. 6, fig. 1 and 2) ammonium acetate (Pl. 6, fig. 3), and ammonium tartrate (Pl. 7, 8, 9, and 15). The relatively small size of the cells in Plates 7 and 8, as compared with the excessively large cells figured by Dr. von Schrenk from his cauliflower intumescences, need not excite comment, since in Plate 9 and also further on we shall see that the cells of the intumescences which I have produced while often smaller are also sometimes much larger than corresponding normal cells of the plant and frequently vary among themselves within wide limits, even in the same intumescence, just as they do in bacterially induced crown galls. It also appears to make a difference what chemical is used or else different volumes of the same substance act differently, large cells being formed under one stimulus and small cells under another stimulus.

Serial sections cut from green tomato fruits exposed to the ammonium tartrate show that the internal proliferations have arisen in many cases at least from a single-layered epithelium in which and under which there is no evidence of wounding. In the same way under the numerous proliferations obtained on tomato carpels with the ammonium acetate I could find no evidence of any cell injuries. In the strengths used the

tissues reacted least to the most injurious substances—that is, to ammonium oxalate, ammonium chlorid, and ammonium nitrate.

I then determined on these plants the action of various dilute pure acids, the same being distilled water solutions of the acid component of the salts already tested, viz, uric acid, carbonic acid, tartaric, citric, acetic, butyric, lactic, oxalic, formic, succinic, salicylic, sulphuric, hydrochloric, and phosphoric acids.

It would take too long to describe in detail all of the experiments with acids which consumed a whole summer and were also unexpectedly positive—that is, showed quite clearly that galls or intumescences may be caused by the action of many dilute acids, including such as various bacteria, larvæ of gall flies, and other parasites are able to produce, and that the previous response to the ammonia salts was not exclusively a response to the alkaline element in them but might be a response due to the combined or joint action of these substances on cells young enough to respond to it.

Very fine intumescences were obtained in this way by injecting 10 per cent solutions of the organic acids—that is, uric acid, malic acid (Pl. 10), citric acid, and tartaric acid. Often the whole wall of the pith cavity was covered with them, and there were frequently large outgrowths around the needle wound where it entered the pith cavity (Pl. 11). But also I obtained intumescences with sulphuric acid and with phosphoric acid and sparingly even with distilled water. These results were unexpected and at first disconcerting, it must be confessed, for I had not then read Loeb's latest remarkable book on parthenogenesis, wherein are detailed many positive and splendid results with acids on animal eggs (15), and which would have advanced my work by at least two years had I read it when it first appeared. I was looking for a specific chemical effect as tumor cause and I discovered instead a general physical (osmotic) effect as cause. I will not undertake to explain the specific action on the cell of all these various substances, since Loeb has speculated on this subject better than I can hope to, and since I have seen nothing to contradict his hypotheses. Much remains for the future, so far as an understanding of the exact mechanism of cell division is concerned, but Loeb seems to have made out quite clearly that the stimulus which sets unfertilized eggs to growing is a purely physical one—that is, an increase in the osmotic pressure of the solutions in which they are placed causes loss of water from the egg surface with the formation of a membrane, which is the beginning of cell division. I believe increased local osmotic pressure also explains the results I have obtained, as well as those which occur naturally in tumors. It is the beginning, I think, of all tumor growth.

If these things are so, why, then, does not the injection of any organism produce a tumor? This is a proper question and may be answered tentatively as follows: (1) In many cases the injected bacteria do not

grow, and therefore the products of their metabolism do not come into play; (2) most of the parasitic forms cause a loss of turgor, requisite for cell-division, either (*a*) by obstructing water-conducting channels, causing the plant to wilt, or (*b*) by excretion of plasma-killing toxins which pave the way for the bacterial advance into the tissues, or (*c*) by excretion of enzyms or other solvents of the middle lamellæ of the cell-wall, in which case we have a softrot—that is, a complete or partial disintegration of the tissues; (3) moreover, the case is not so unique as some persons suppose, because various other bacteria than the crown-gall organism do actually produce overgrowths, large or small—that is, granulomas (often of large size), tyloses in vessels or small surface intumescences (I know of half a dozen or more such bacteria and new ones are coming to light every year); (4) a very considerable number of fungi also cause overgrowths; (5) the number of insects that cause overgrowths is legion.

I will only say here, further, that there is really no reason to be disturbed by the variety of substances shown to be capable of causing cell proliferation, since probably only such substances can cause neoplasms as are thrown off locally and continuously into the tissues by cancer organisms, and that too *intracellularly*, for otherwise only granulomas should result in plants, while in animals the blood stream would quickly remove and effectually dispose of any irritating substances not continually replenished. Moreover, in animals the tissue naturally most exposed to foreign substances—viz, the epithelium—is by nature the most resistant—that is, the last to yield to cancerous proliferation.

Having come to the conclusion that I was dealing with a general physical problem rather than with a specific chemical problem, I next tried the effect of injecting various foods and poisons as follows:

Ten per cent tannic acid (Pl. 11), 10 per cent ethyl alcohol, 2 per cent sodium chlorid, 2 per cent sodium carbonate, 5 per cent sodium bicarbonate (Pl. 12), *N/20* sodium hydroxid, 5 per cent ammonium bicarbonate (Pl. 13), clear lime water (Pl. 14), milk of lime (caustic), 1 to 10,000 mercuric chlorid, 0.5 per cent carbolic acid, chloroform water, 1 to 1,000,000 copper sulphate water, 5 per cent grape sugar (Pl. 14), 5 per cent cane sugar (Pl. 15).

These experiments also yielded positive results and very striking ones in some cases, even the grape-sugar and cane-sugar ones.

Next, I undertook to determine the effect of very minute doses of ammonia—doses so weak that surface woundings were not to be expected. For this purpose I used the feeble alkaline vapors arising from dilute solutions of urea, ammonium carbonate, and the two ammonium phosphates (Pl. 16 and 17), these substances being inclosed in open small test tubes sealed into the pith cavity of Ricinus stems by means of collodion or surgeons' adhesive strap. I also injected 1 to 100 and 1 to 1,200 ammonia water with striking results (Pl. 18–31). Subsequently

I inserted tubes containing stronger solutions of monobasic ammonium phosphate (20 per cent) and from its vapors obtained many positive results (Pl. 32–36). All of these experiments yielded positive results—that is, fine proliferations from surface cells of the pith cavity, *not only in the opened internodes, but also in many unopened ones above and below*, and consequently where the action had to take place at a distance through thick cross walls.

From these experiments there can be no reasonable doubt, I think, that any soluble substance whatsoever, except a killing, a plasmolyzing, or an oxygen-absorbing substance, if continually liberated in excess locally in tissues would be competent to induce tumor formation.

One of the most striking results of these experiments has been *the production of a stem within a stem.* Recently in THE JOURNAL OF AGRICULTURAL RESEARCH (Apr. 24, 1916) in detailing various experiments made with the crowngall organism I showed that under some circumstances the bacterial stimulus caused the production of a stem within a stem (21, Pl. 21). The plant used was the common tobacco (*Nicotiana tabacum*), and the new stem, or more properly *stele* (which I called a "tumor strand" because at frequent intervals it induced the formation of secondary leafy tumors), was developed in the outer cortex of the normal tobacco stem between tumors where I believe no such structure was ever seen before.

I have now been able to produce this strange phenomenon of a stele within a stele in the absence of the crowngall bacterium by simply injecting one of its products—to wit, an ammonium compound—into growing tissues. Internodes of the castor-oil plant (*Ricinus communis*) were used for all of these experiments. I selected this plant rather than tobacco because it is easier to work with than a plant having a solid stem, and also because the fluids could be injected in any desired small quantity without tearing the tissues, the only mechanical injury being the small hole made by the hypodermic needle.

The results obtained have been very surprising and are, I think, quite suggestive not only of the growth previously obtained between tumors in a tobacco stem by means of the bacteria (the cortical stele formation), but also of the tumor formation itself. The photomicrographs show my results very clearly and only a few explanatory words are necessary. These results were obtained with the monobasic ammonium phosphate. Young, half-grown, and old internodes were injected but striking results were obtained only once, when a very young internode was injected.

The manner of experimentation was very simple. The needle of a glass-walled hypodermic syringe was thrust through the upper part of the wall of the internode and the cavity (very small in the young internode) was filled with distilled water in which 5 per cent of the ammonium salt had been dissolved. Sometimes the needle wound was closed with collo-

dion, but this is not necessary. Needle wounds without injection—that is, where only air entered, gave no such results; neither did the injection of pure water. The bark and wood cylinder were not visibly affected by the injection—that is, there was no change in color and no distortion or abnormal increase in the diameter of this part of the stem—but the pith, in contact with the solution, proliferated into the pith cavity enormously as a compact cylinder of small cells narrowing the lumen almost to closure, especially at the upper end, the diameter of the stem wall being increased to more than double its normal thickness—that is, from 3 to 7 mm. The normal Ricinus stem of this age contains a large pith cavity and a relatively thin wall. The xylem-phloem cylinder in such a stem is well toward the surface, the cortex being thin and the pith relatively thick. (Pl. 37, fig. 1.) The bundle is not bicollateral.

Adding the proliferated pith tissue to that normally present gives a very thick cylinder of pith in the inner part of which, entirely surrounding the pith cavity (source of the stimulus), a second xylem-phloem cylinder has developed. (Pl. 37, fig. 2.) About the tenth or twelfth day death occurred in the middle part of the thickened pith cylinder as shown by the appearance on cross sections (Pl. 37, fig. 2) of a wide white ring midway between the two complete xylem-phloem cylinders, indicating substitution of air for water, and soon after the inner xylem-phloem cylinder was torn from the outer part of the pith, probably by cessation of growth on the part of the inner pathological cylinder, the stimulus from the pith cavity having been exhausted, with continued growth of the outer xylem-phloem cylinder. The inner cylinder then lies loose in a large cavity, like the skin of a caterpillar in a cocoon, or like a rough-coated stick of macaroni (Pl. 37, fig. 3), the surface being covered with coarse shreds of the dead, white pith. The interior of this cylinder is still living and the inner face is covered with a sheet of living, loosely connected, rounded, glistening, turgid cells—the final proliferations in response to the waning stimulus. The wall of the outer inclosing stem has now about the same total thickness and relative arrangement of tissues as the whole stem had at the beginning of the experiment (compare fig. 2, 3, and 4 of Pl. 37) except that the pith is narrower (approximately one-fifth cortex, two-fifths xylem-phloem, two-fifths pith); but its inner face is covered with coarse white flecks of dead pith and all its inner cells are more or less shriveled. The pathological stele in the tobacco stem occurs in the outer part of the fundamental connective or conjunctive tissue (the cortex); and this pathological stele in Ricinus occurs in the pith, another part of the same fundamental tissue.

This second xylem-phloem contains primitive spirals, tracheæ, wood fibers, cambium, and sieve-tube tissue, the numerous bundles that compose it being regularly separated by medullary rays. (Pl. 38–41.) This pathological stele within the normal stem has a reversed polarity—that is, its spiral vessels face those of the normal xylem cylinder but are

separated from them by a wide tract of pith, while its phloem faces the pith cavity but does not come to its surface. Within itself the tissues of this abnormal xylem-phloem cylinder are arranged in a fairly orderly manner, but not as perfectly as in the normal outer xylem-phloem cylinder—that is, (1) there is an increased number of bundles, (2) the conjunctive tissue between the bundles is narrower and its component cells are smaller than in the normal xylem-phloem cylinder, (3) the cambium ring is thicker, (4) the xylem-phloem vessels are fewer, and (5) the wood fibers are more numerous. There are also many distortions strikingly suggestive of crowngall—that is, twisted bundles, spiral vessels, and tracheæ lying diagonally or at right angles to the longer axis of the stem, and loops and distortions of the cambium (toward the pith cavity) leading in places to the formation of islands of xylem-phloem (well-staining tracheæ and occasional sieve tubes) inside the second xylem-phloem cylinder close to the pith cavity (Pl. 39–41), as if the plant when cut was in process of constructing other xylem-phloem cylinders more imperfect than the second because the stimulus was exhausted. The tissues in the imperfect third cylinder face the reverse way from those of the second cylinder, while those in the fourth face the same way. Beyond these is also a cambium without accompanying vessels (broken dark line, Pl. 38, fig. 1).

Great thickenings of the pith with the same cylindrical tearing loose of the inner (proliferated) part from the outer (normal) pith were also obtained with ammonia (Pl. 29, fig. 1), with dibasic ammonium phosphate (Pl. 42) and with other substances, but no perfect double cylinder of xylem-phloem. I did obtain, however, in a young internode next above an injected one ($\frac{1}{100}$ ammonia) a dislocation of a few vascular bundles extending the entire length of the internode. These bundles are buried in normal looking pith (Pl. 20) which forms a very decided ridge projecting into the pith cavity. Here the general appearance strongly suggests the pathological stele obtained in the tobacco stem, but the orientation of the xylem-phloem is exactly the reverse of that in the tobacco stele. In that there was an outer hollow cylinder of phloem, a middle hollow cylinder of cambium and an inner cylinder of xylem. In this Ricinus stele the outermost hollow cylinder is xylem, the middle hollow cylinder is cambium, and the central solid cylinder is phloem (Pl. 43). If, however, we consider that part of each stele which faces the normal xylem-phloem cylinder they are alike—that is, they follow the general law of duplication of parts—viz, that the excessive part is a mirror image of the normal part.

Moreover, the structure of these dislocated bundles is curiously like the whorls of tissue (tracheæ, etc.) that occur in the tumor-strand or fundamental tissue in the interior of secondary crowngalls produced by *Bact. tumefaciens*. I have frequently figured (4, Pl. 36, 39, 40, 83, 84; 18, Pl. 8, 12, fig. 48) and referred to these interesting whorls, but have

not hitherto described their component parts. They are within the vascular cylinder of the tumor in what I take to be modified pith and they also have the same order and arrangement of parts as the Ricinus steles just described—viz, spiral vessels on their periphery followed by tracheæ and cambium within which are sieve tubes, and sometimes in the larger ones a small amount of the fundamental tissue forming a coarse-celled center.

I observed the same phenomenon in another Ricinus stem the pith cavity of which had received ammonia. Here no less than eight concentric medullary bundles (phloem strands surrounded by xylem, as in crowngalls) developed in the outer pith near the vascular cylinder (Pl. 44 and 45) at one level and 16 at another level in a circumference of less than 1 cm. In this connection, however, see Plate 65.

Furthermore, in studying the outer part of the same cross sections (middle part of the Ricinus internode shown on Plate 5) I discovered under the dead pith cells at either side of the proliferating cells, shown in the center of the picture, cushions of proliferating tissue derived from the inner (parenchymatic) face of the uninjured xylem bundles and in the innermost part of these cushions close to the layer of killed tissue a small group of phloem cells under which (that is, farther from the pith cavity) are spiral vessels, with what appears to be cambium between them (Pl. 46). In other words, here is another example of a pathologically induced second xylem-phloem cylinder, much more imperfect than that produced by the ammonium phosphate but oriented in the same way—that is, it is the reverse or mirror image of the normal xylem-phloem, but is separated from the latter by a considerable abnormal growth of parenchyma. In Plate 47, figures 1 and 2 show cross sections of the central strongly proliferating part of Plate 5. Only tissues near its outer part are here shown, but in them are tracheæ and twisted cells strongly suggestive of crowngall distortions, while deeper in (Pl. 45, fig. 1) are the normal elements of the bundle. Calcium-oxalate crystals are very numerous in and near the killed pith tissues, are not elsewhere present in the sections, and have never been seen by the writer in normal Ricinus pith of this age. This suggests that normally in dividing cells ammonia is perhaps the first term in a series of reactions leading, in the plant, to the production of oxalic acid, which, as we know, is an almost universal product of its growth.

In one case also where limewater was injected I succeeded, in the lower third of an internode, in closing the pith cavity altogether with normal pith proliferations in which no wound tissue is visible (Pl. 14, fig. 3), but no second cylinder of xylem-phloem was formed in it; nor have I thus far succeeded in reproducing the phenomenon with the monobasic ammonium phosphate. My later experiments, I now believe, were done on internodes which were too old.

These results and recent ones with crowngall teratoids (bacterially induced embryomas) lead me to believe that not only the origin of fasciations but also of many other duplications is to be sought in local and feeble infections by a variety of deep-seated microorganisms.

Duplicate vascular cylinders in the pith and scattered concentric cortical or medullary bundles do not occur normally in the internodes of Chrysanthemum, Nicotiana, Ricinus, or any of the higher plants I have worked with, so far as I have observed, but triple sheathing cylinders of xylem-phloem (the inner ones standing in pith) occur normally in some of the ferns (Matonia). Scattered concentric medullary bundles (with a central phloem) occur in the stems of various dicotyledons, but are regarded as anomalous, while concentric cortical bundles are said to be rare (Solereder). Some of the phenomena here described are therefore possibly to be regarded as in the nature of reversions to earlier and more primitive stem structures or else as indications of premature development of tissues which are normally developed only toward the end of the life cycle of the plant. Since this paragraph was written I have found a paper by Martin Möbius (25) describing and figuring a concentric bundle with central phloem from the axis of inflorescence in *Ricinus communis*. This stimulated me to examine sections from numerous nodes and internodes of Ricinus, as well as from the tap root, hypocotyl, cotyledons, leaves, and axis of inflorescence with the following result:

Concentric medullary bundles with phloem at the center are very numerous in the axis of inflorescence (Pl. 65, fig. 1); they occur sparingly in the nodes, and are present in branch-gaps immediately above the node (Pl. 65, fig. 2). I did not find them elsewhere in the internodes. They were not seen in the taproot, hypocotyl, cotyledons, petioles, or leaf-gaps.

Möbius gives reference to earlier literature on concentric bundles of this type, especially Russow (23) and Bergendal (24), and at the end of his paper a list of families in which they have been observed.

I have not been able to find such bundles in any part of the Paris daisy, although they occur in the crowngall on this plant and normally in the pith of certain compositæ (*Scorzonera hispanica* and *Tragopogon pratensis*).

Finally, I repeated Dr. von Schrenk's ammonia tests on cauliflower, obtaining numerous magnificent proliferations which could only be attributed to the ammonia used, since no copper salts entered into the experiments. At first I painted on dilute ammonia, but later and much more satisfactorily (Pl. 48, 49) I exposed the plants for a few minutes in a tight box containing 10½ cubic feet of air space to vapors from a small quantity (1 c. c., ½ c. c., $\frac{1}{10}$ c. c.) of aqua ammonia (0.90 sp. gr.). The vapor undoubtedly entered through the stomata which are chiefly

on the under surface, and the outgrowths are almost entirely from the loose parenchyma of the leaf (Pl. 50, 52). In structure these tumors are either pure hypertrophies or mixed hyperplasia and hypertrophy (Pl. 50–54).

Also, I obtained numerous fine intumescences on cauliflower leaves by exposing them for a short time to mixed vapors of ethyl alcohol and acetic acid (Carnoy's fixing fluid), but only when the stomata were nearly closed (presumably). In maturer rapidly transpiring leaves on the same plant the effect of the vapor was entirely different—that is, there was a localized killing effect, giving to the leaves a curious mottled green-and-white appearance (Pl. 55). I looked in vain on such leaves for intumescences. There was not a trace even on the margins of the white spots. These leaves were in full growth and vigor, not old leaves. Those leaves which developed the intumescences were younger leaves, only partly developed at the time of exposure (Pl. 56). These would have their breathing pores closed or nearly closed at noon, and consequently only a stimulating minimum of the vapor could enter. The appearance of these growths in fresh vertical sections is shown on Plate 57 and in cross and vertical sections from fixed and stained material on Plates 58 to 61. They are quite unlike most of the ammonia intumescences in structure, although derived in good part from the same tissues of the leaf (loose parenchyma)—that is, the ammonia intumescences, except two old ones, are made up almost exclusively of a few hypertrophied cells (Pl. 52–54), while these are composed of many intermingled small to moderately coarse cells, much more suggestive of a crowngall hyperplasia, than which, however, they are more regular. They also suggest incipient shoots—that is, growing points, and those 7 days old show the beginnings of a vascular system derived from the smaller vascular bundles of the leaf (Pl. 60, 61). I believe they are actually pathological growing points, and if so they are a striking example of the ability of the cauliflower leaf to develop totipotent tissues (embryomas) from many parts of its under surface when properly stimulated. The cells of these intumescences form a compact tissue that is very unlike the loose parenchyma from which it has been derived. There is, therefore, a suggestion in these results that one chemical substance may induce hyperplasia of cells and another hypertrophy, both of which phenomena occur in crowngall and in various animal neoplasms. Still other substances are perhaps required to produce multinucleate cells.[1]

[1] As distinguished from hyperplasia three striking instances of hypertrophy of cells in connection with tumor growths may be referred to here: (1) The root nodule of legumes due to nitrogen-fixing bacteria, (2) the finger and toes of turnips and cabbages due to a spore-producing myxomycete, (3) galls on the roots of a great variety of plants caused by parasitic nematodes of the genus Heterodera. In the first and second cases the enormously enlarged cells are densely occupied by the parasites which destroy the cell contents, plasm and nucleus, and consequently these cells are not giant cells in the meaning of the animal pathologist, namely, not multinucleate cells. On the contrary, the enormously enlarged cells in the nematode galls should be of special interest to animal pathologists, because they are true giant cells, their development

Similar results were obtained on cauliflower leaves by exposure to the vapor of secondary methylamine (Pl. 46, 62, 63, 64).

Also on cauliflower plants exposed to the vapor of primary ethylamine the younger leaves showed distortions and thickenings of the parenchyma with changes in the palisade tissue quite suggestive of curly top of sugar beets, mosaic of tobacco, and similar diseases of undetermined origin but suspected of being due to parasites (Pls. 63, 64). The etiology of these obscure diseases can perhaps be unraveled by exposure of healthy plants to the products of a variety of microorganisms and here, possibly, is a clew.

Consequently without entering into further details I will say that I think I have established my original hypothesis—viz, that dilute ammonia causes intumescences and have rendered it probable that ammonia liberated within the cell in small quantities by the imprisoned bacteria must be one of the causes of the excessive and abnormal cell proliferation in crowngall. Probably amin compounds also help to determine it. Since an acid and an alcohol are likewise produced by the crowngall bacteria and this alcohol and this acid (as well as many other acids) in pure dilution and also in combination with ammonia caused galls or intumescences in my experiments, the acid (or acids), the alkalies, and the alcohol must, I believe, act together in producing the tumors, and *osmotically rather than chemically.*

To return now to the experiments as a whole, here is abundant further evidence that changes in stimulus can produce changes in structure, if any such additional evidence were needed, considering the multiplicity of arguments from regeneration experiments, especially on animals. And this time the changes, some of which if more regular (and there is a reason for that) are nevertheless as striking as anything observed in beginning neoplasms, have been produced, in the absence of bacteria, with known chemical substances—some of them the by-products of the metabolism of a cancer parasite, provided, of course, that crowngall is admitted into the family of the neoplasms, as I think it must be (18, 19, 20).

out of ordinary cells being in some way brought about by a closely attached but external parasite which feeds upon them as they grow by thrusting its mouth parts into them These cells are often 20–30 nucleate and a hundred times as large as the normal cells, so that often in sections of the galls they have been mistaken for parts of the parasite, being quite unlike anything ever observed in the normal tissues of the host. Here the stimulating substance, which might well be some ammonia compound from the urine or feces of the feeding larvæ, not only stretches the cell wall, as in 1 and 2, but that or some other substance also causes a fission of the nucleus. But fission of the nucleus may occur in crowngall without any marked enlargement of the cell, and also cells in that tumor may show hypertrophy without developing multiple nuclei. When discussing my results with Dr. N. A. Cobb, of the Bureau of Plant Industry, he called my attention to certain facts respecting the orange-root nematode of Florida, which seem to me to be very pertinent in this connection. This eelworm, which is the type of his new genus Tylenchulus (16, 17), does not produce galls, although it is closely related systematically to the gall-forming eelworms of the genus Heterodera. This striking difference is correlated with the fact that the larvæ of Tylenchulus are hatched free in the earth and never insert into the roots of the orange plant anything but their head parts, all their anal excretions being voided into the earth, whereas the larvæ of Heterodera are entirely buried in the plant from the beginning, so that all their excretions must necessarily come into intimate contact with the growing tissues of the host. It would seem, therefore, that nematode galls must be due to anal rather than buccal or skin excretions.

No unbiased person, it seems to me, remembering that I have done these things with a fleeting stimulus (fluid or vapor) applied entirely from a cavity (stomatal, carpellary, or pith) and not acting from within the cells as would be the case in cancer, can look at the striking cell proliferations which I have obtained and which are so like early stages of crown-gall without being convinced that the growths would have gone on indefinitely, with the formation of large irregular tumors, rupturing to the surface if the chemical substances leading to the increased osmotic pressure could have been applied slowly, continuously, and in varying localities as must be the case when the by-products of intracellular proliferating bacteria are the cause of the tumor. Moreover, if wood and bark and organ fragments of all sorts (18, 21) can be developed out of place in plants by a local stimulus why not also in the same way cartilage, bone, muscle, and fœtal fragments out of place in animals?

To conclude, it would seem, therefore, that in local osmotic action (possibly in some stages chemical action also) of various substances (aldehyde, acetone, alcohol, acids, alkalies) thrown into cells and diffusing from them in various directions, as the result of the metabolism of a feeble intracellular parasite or symbiont together with the resultant counter movements of water and food supply we have, in crowngall at least and presumptively also in animal neoplasms, the explanation of tumor growth—that is, of that extensive multiplication of cells in opposition to physiological control which has so long puzzled pathologists and all students of overgrowths.

SUPPLEMENTARY REPORT OF THE CHEMIST.

[This report was received too late to be inserted in its proper place in the text It should be read in connection with the Chemist's Report, on page 169]

Flasks N, O, P, and Q, cultures of the hop strain of *Bacterium tumefaciens* inoculated on November 4, and flasks R and S, checks on the hop, were received from Dr Smith for analysis

Flasks N, O, and P were opened on January 15 (72d day), their contents united and distilled to get rid of neutral volatile products This distillate gave a positive test for aldehyde Alcohol and acetone were absent

The residue being reduced to about half its original volume was treated with 75 c c 5*N* sulphuric acid and distilled with steam The distillate was neutralized with barium hydroxid, evaporated to dryness and the crystalline residue dissolved in water and filtered The filtrate was treated with zinc sulphate. This precipitated the barium as sulphate, which was filtered off The clear filtrate containing the organic zinc salts was evaporated to a very small volume and taken up in alcohol Zinc acetate is soluble in alcohol, while zinc formate is insoluble A considerable precipitate showed the presence of zinc formate This was confirmed by dissolving in water after filtering and boiling with a few drops of silver nitrate, when metallic silver was deposited as a mirror

The filtrate was evaporated to dryness and taken up in just enough water to dissolve it. On adding silver-nitrate solution a brown precipitate formed, which turned black on boiling, indicating formic acid. Acetic acid was not found in these cultures

Jan 16, 1917 (Signed) J F. BREWSTER.

Plates were poured from each one of these flasks before turning over to the chemist and appeared to be pure cultures of the crowngall organism. Plants have also been inoculated with subcultures from colonies taken from these plates, but it is too early to know results.

The bacterium used for these flask cultures was the original hop strain kept for a long time in the laboratory without passage through plants (9 years), but was still infectious. That used for the first set of hop flasks (which were only 2 weeks old) was the same strain but after passage through sunflower in 1915

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PLATE 4

Pith cavity of a stem of *Ricinus communis* showing proliferations of deep origin due to the injection of a mixture of strong ammonia and water (aqua ammonia, sp. gr. 0.90, 1 part, and distilled water 10 parts). The surface of the pith, as at X, X, is dead. The proliferations are from the inner face of the xylem at the bottom of the internode and the needle wound was about 5 cm. above this point. Injected on June 2. Photographed on July 13, 1916. Time, 41 days. ×14.

PLATE 5

Opposite side of the pith cavity of *Ricinus communis* shown in Plate 4, but more highly magnified to show the individual cells of the proliferations. ×28. For cross sections see Plates 44 to 47. The middle of this figure is about 1 inch above the node.

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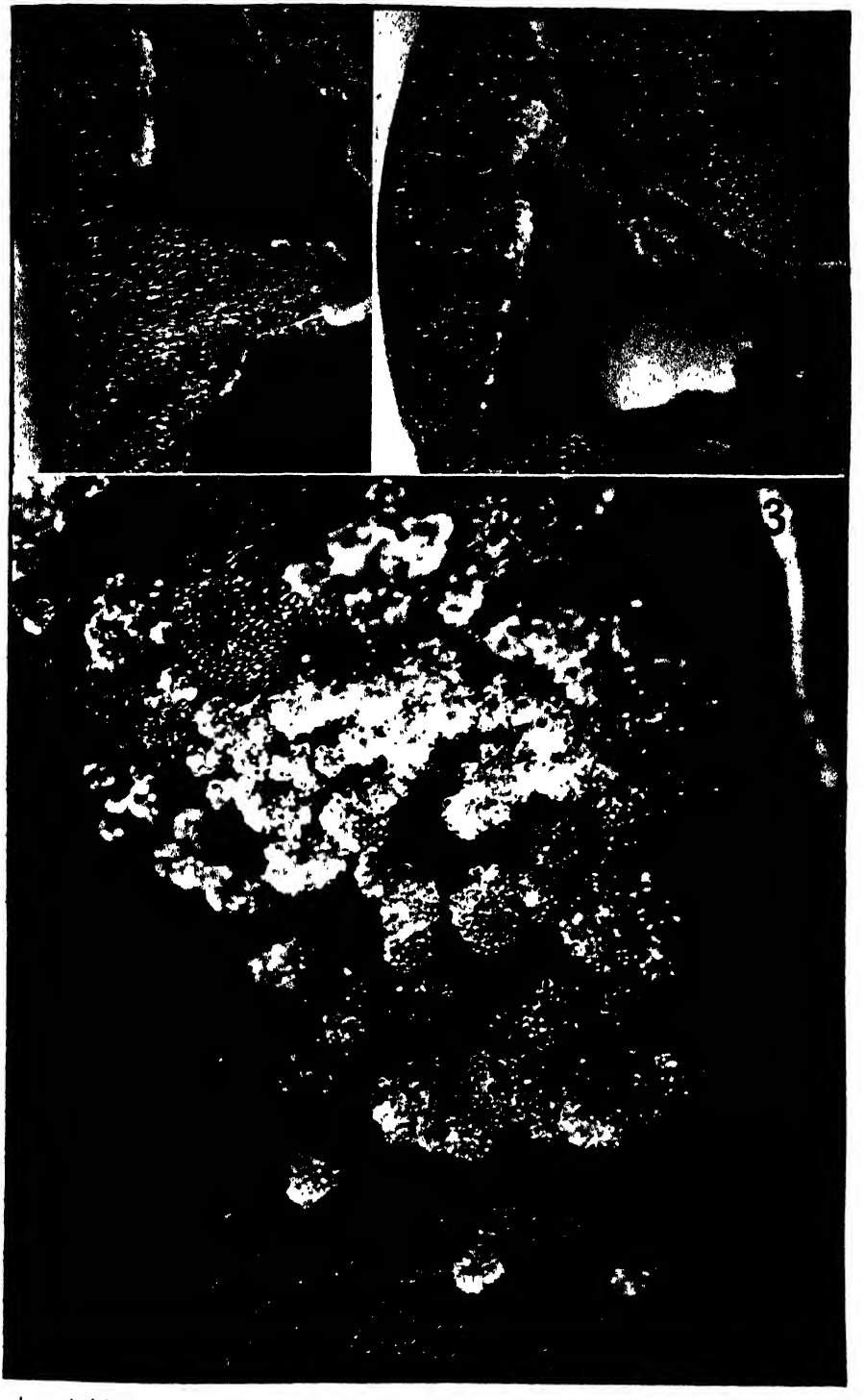
PLATE 6

1, 2.—Sections of a green tomato fruit showing carpellary surface proliferations due to ammonium carbonate (20 per cent solution). Time, 10 days. Injected on June 10. Photographed on June 20, 1916. ×7. It is probable that only vapors from the carbonate touched these places, otherwise there would have been woundings.

3.—Inner face of carpel of a green tomato fruit showing numerous mulberry-like white surface proliferations due to the injection of ammonium acetate (20 per cent solution). Carpel surface uninjured—that is, there were no dead specks in the pale green carpel wall either between these proliferations or under them. Photographed on June 22, 1916. Time, 12 days. ×14.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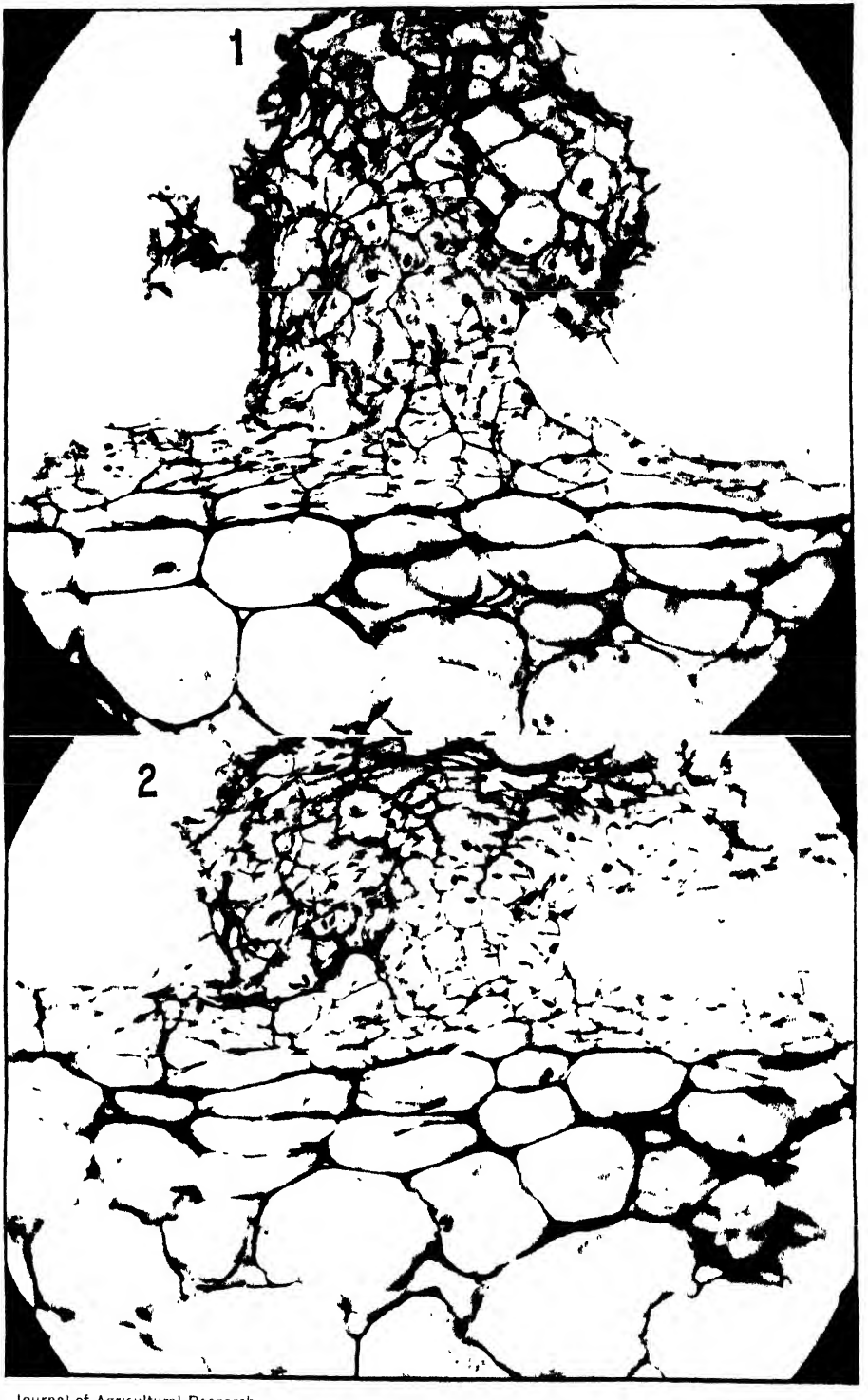
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PLATE 7

1, 2.—Inner face of carpel wall of green tomato fruit showing proliferations due to ammonium tartrate (20 per cent solution). The growths are from the lining membrane which is normally one cell thick, as shown on the right side of the upper figure, and in Plate 8, figure 2. Injected June 10, 1916. Collected on June 27, 1916. Time, 17 days. ×160. Block 1252 B 5. Cells shriveled more or less by the fixative.

PLATE 8

1.—From the same series of sections as the figures on Plate 7, but showing a more copious proliferation of cells. It is clearly a proliferation from the lining membrane. Carbol-fuchsin stain. Starch (*st*) in the deeper cells. The dark bodies in the tumor cells are nuclei. ×160. Block 1252 B 4.

2.—Normal tissue of inner face of tomato carpel in vicinity of figure 1, for comparison with that and with figures on Plate 7. Same magnification.

3.—Lining membrane of figure 2, passing into such a tumor as figure 1. ×160.

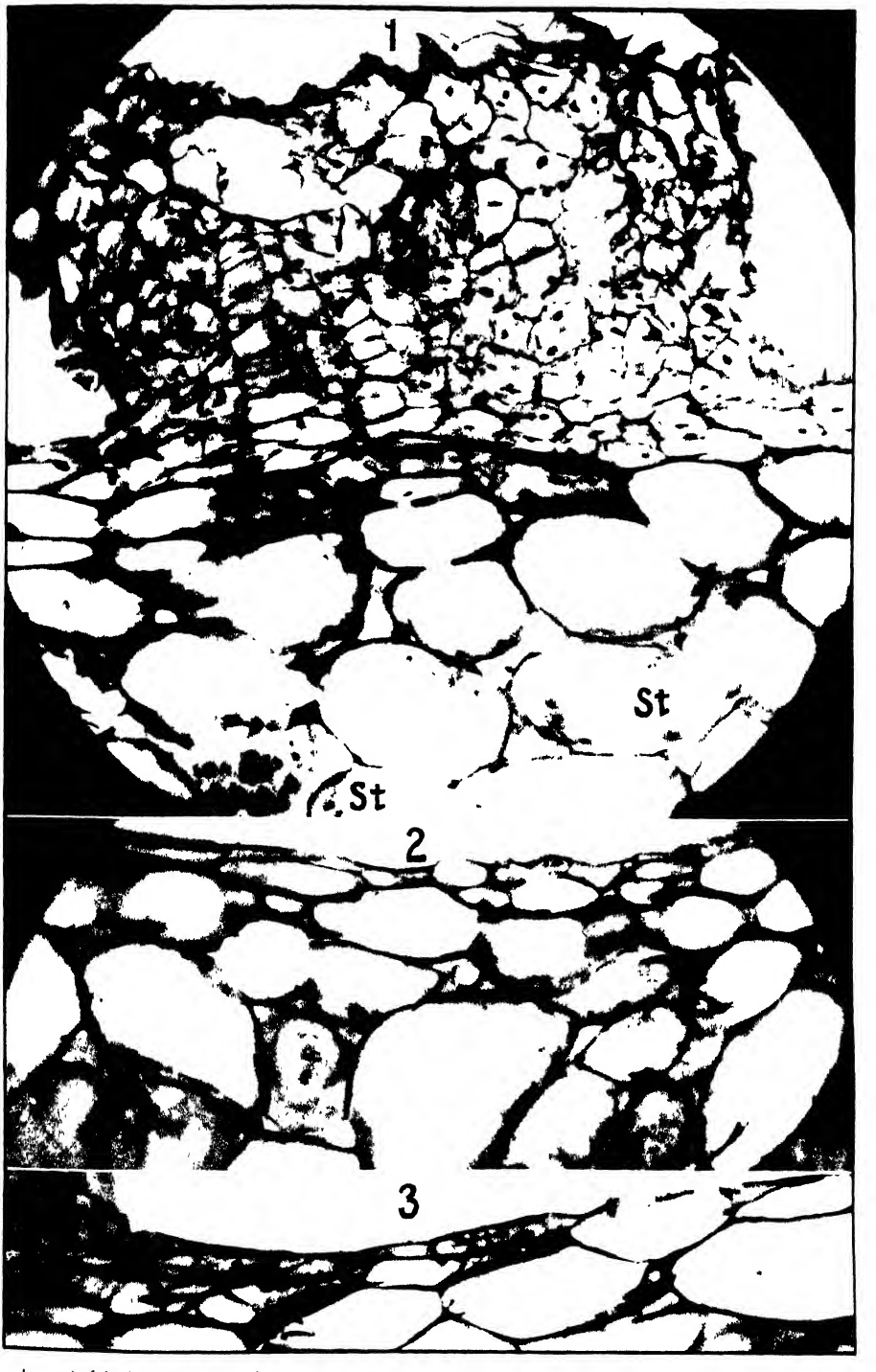
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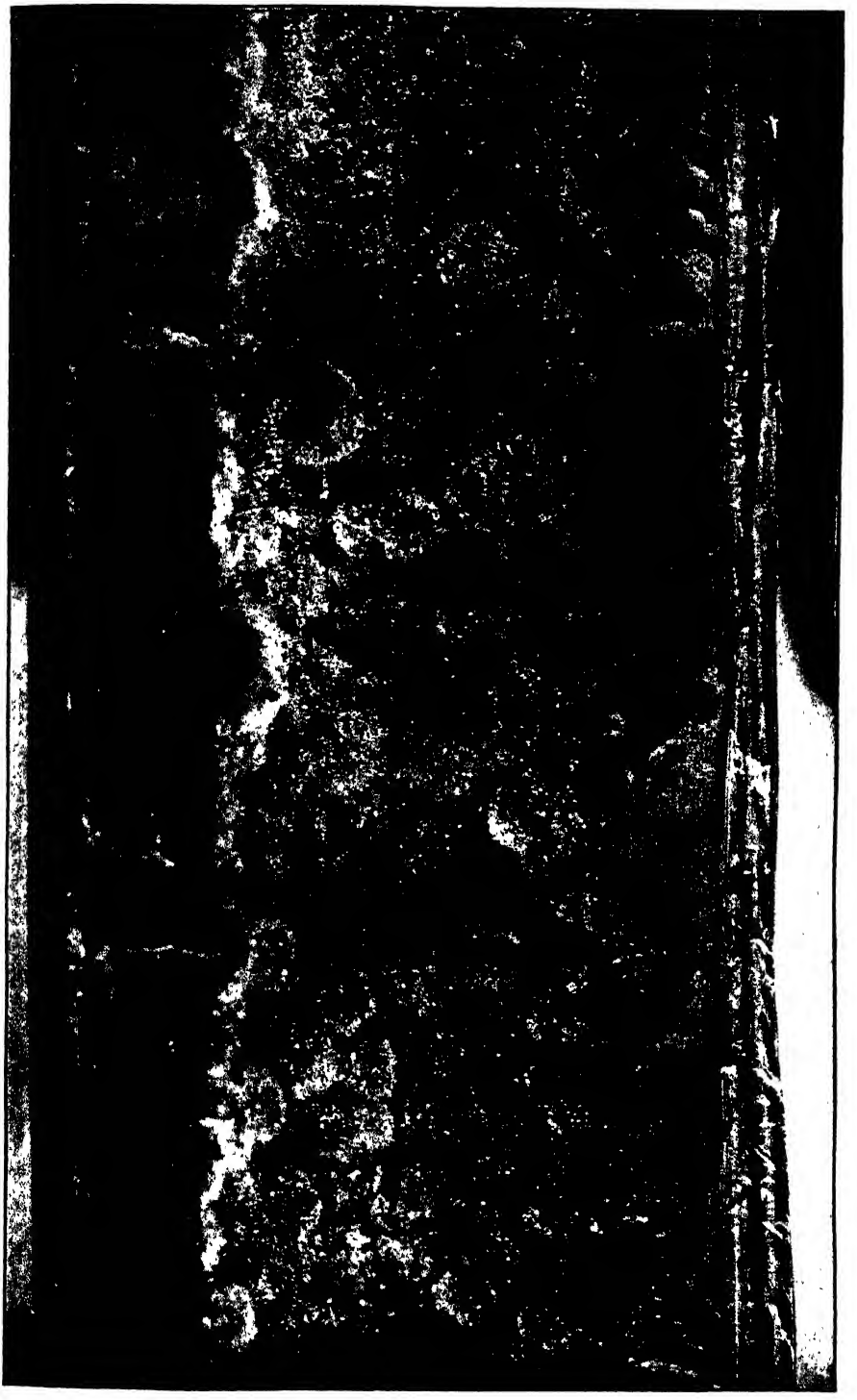
PLATE 9

1, 2.—Same slide as Plate 7, but showing greater variations in the size of the proliferated cells. Figure 1 was made with the 8 mm. and figure 2 with the 16 mm. lens. The tumors have grown wholly from the lining membrane. Block 1252 B 5. ×160 and 75, respectively.

PLATE 10

Longitudinal section of a stem of *Ricinus communis* injected July 18, 1916, with distilled water containing 10 per cent malic acid. Photographed on July 28. ×9. There is a copious proliferation from the outer pith cells filling the pith cavity. Malic acid in small amounts is a normal constituent of the plant cell.



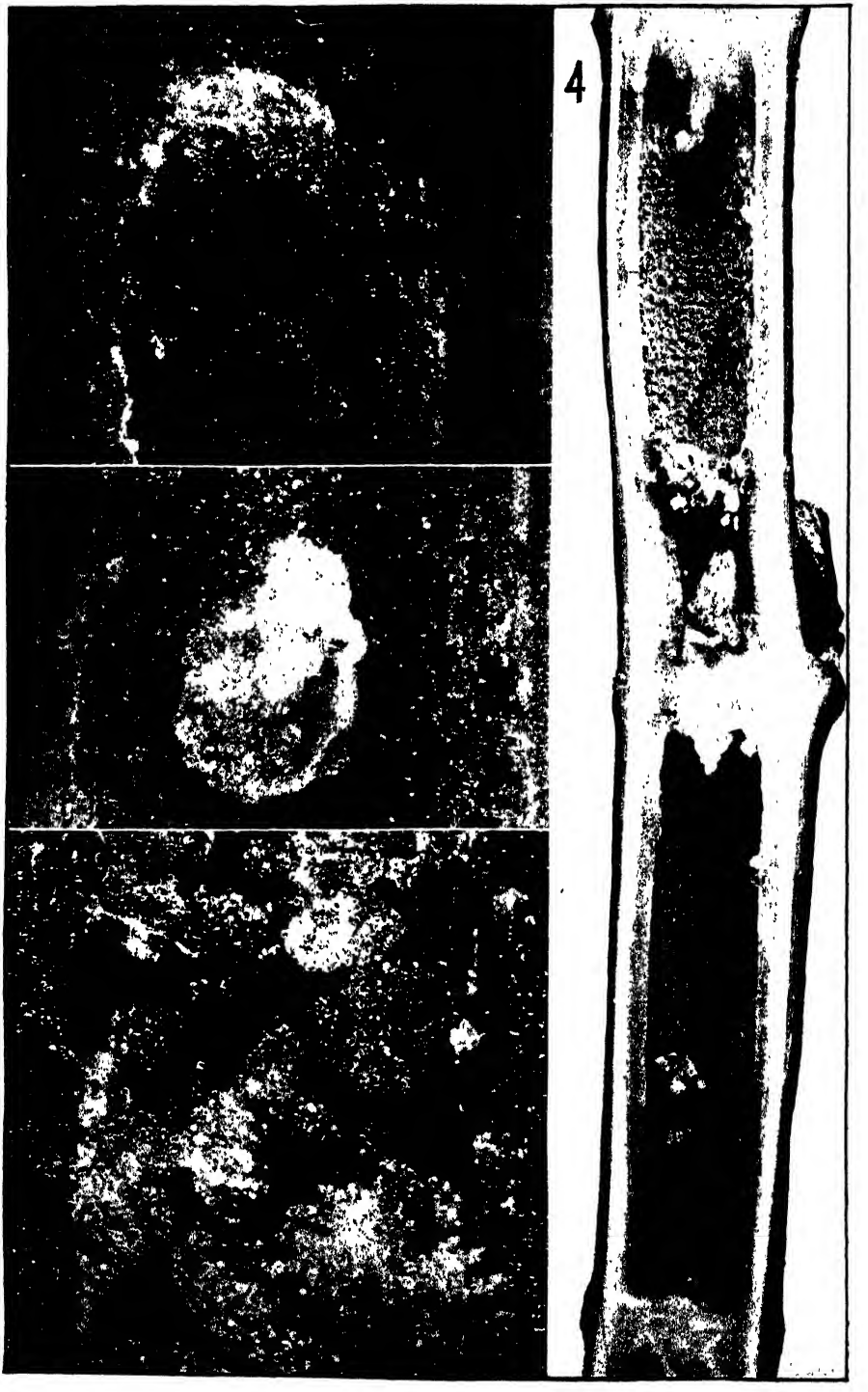

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PLATE 11

1. Middle of an internode of *Ricinus communis* injected July 18, 1916, with 10 per cent malic acid, showing copious surface pith proliferations. The central large nodule developed around the needle entrance. Photographed on July 28, 1916. ×8.

2.—Middle of a Ricinus internode injected July 18 with 10 per cent uric acid. Surface of pith covered with granulations The central large nodule developed around the needle entrance. Photographed on July 31, 1916. ×12.

3.—Middle of a Ricinus internode, showing copious tumor-like proliferations due to the injection of a 20 per cent solution of urea These proliferations were yellowish rather than a pure white, as in most cases. Injected June 29, 1916. Photographed on July 11. ×11.

4.—Two Ricinus internodes injected with a 10 per cent solution of tannic acid on August 7, 1916. Photographed on October 20. Natural size. Pith killed and black in the lower third of each; in the upper two-thirds a continuous sheet of proliferations from the pith surface with occasional nodules. The lower large nodules are wound reactions from deeper tissues. Each ceiling also shows proliferations. The killed part of the lower internode was examined for crystals of calcium oxalate but only an occasional crystal was found, not more than would occur in normal stems of this age (see Pl. 46).

PLATE 12

1, 2.—Internodes of *Ricinus communis* injected August 12, 1916, with a 5 per cent water solution of sodium bicarbonate. Cavity filled with copious wound proliferations, all of which are from the pith. The light-colored longitudinal band on either side of the pith cavity shows the extent of the thickening. Killing effects as fissures are visible here and there in figure 1, especially in the inner (sectioned) part of the pith on the right side and more abundantly in figure 2 as caps on the nodules. Photographed on August 25. ×5.

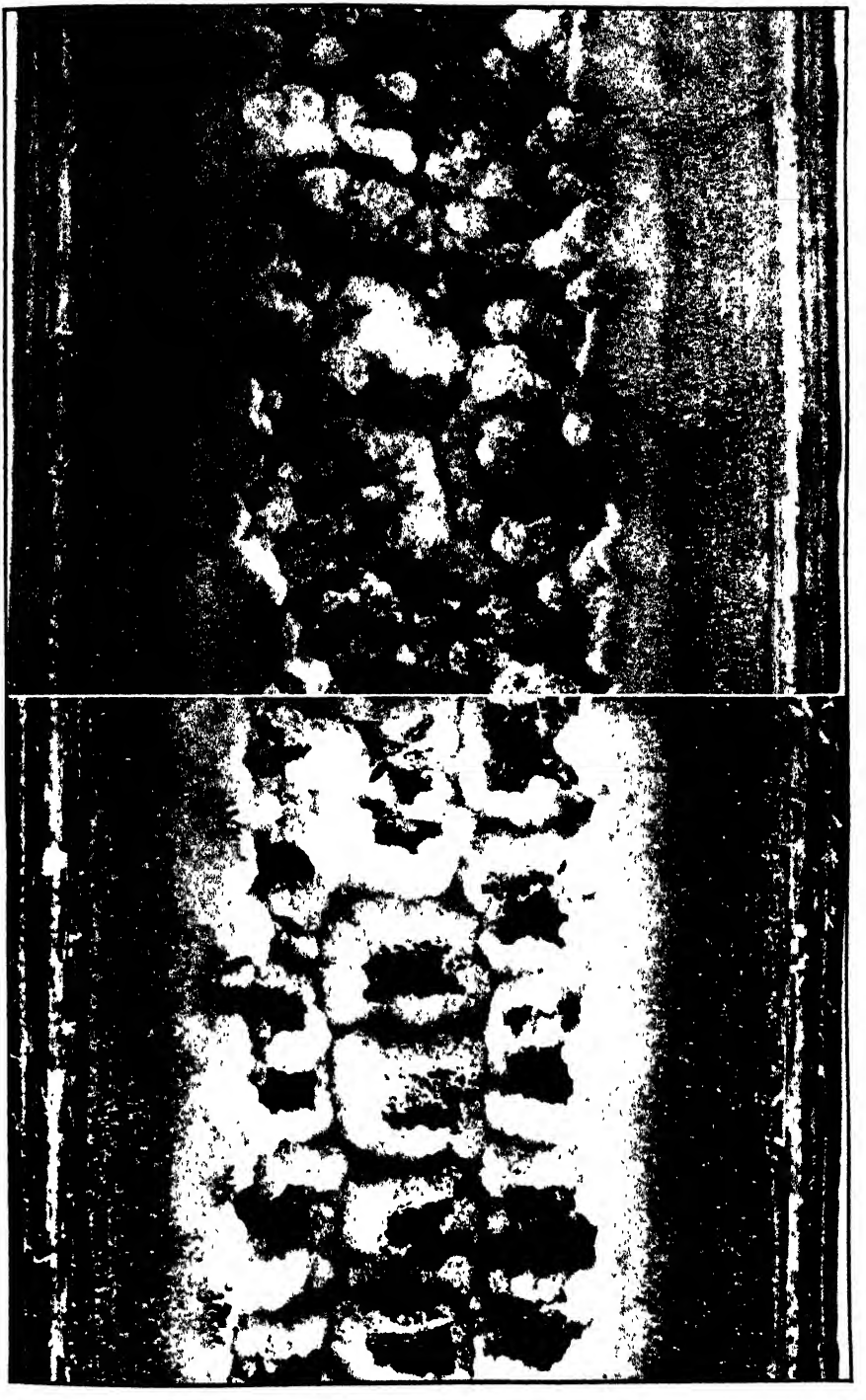

PLATE 13

Internode of *Ricinus communis* showing a pith cavity occupied by numerous anastomosing, white glistening strands and small nodules lying loosely connected on the surface, and due to injection on August 12, 1916, of a 5 per cent solution of ammonium bicarbonate. This is an unopened internode—the second internode above the uppermost injected one. Portions of the injected internodes were killed. For the normal appearance of such an internode see Plate 37, figure 1, and branches of Plate 14, figure 1. Photographed on August 25. ×5.

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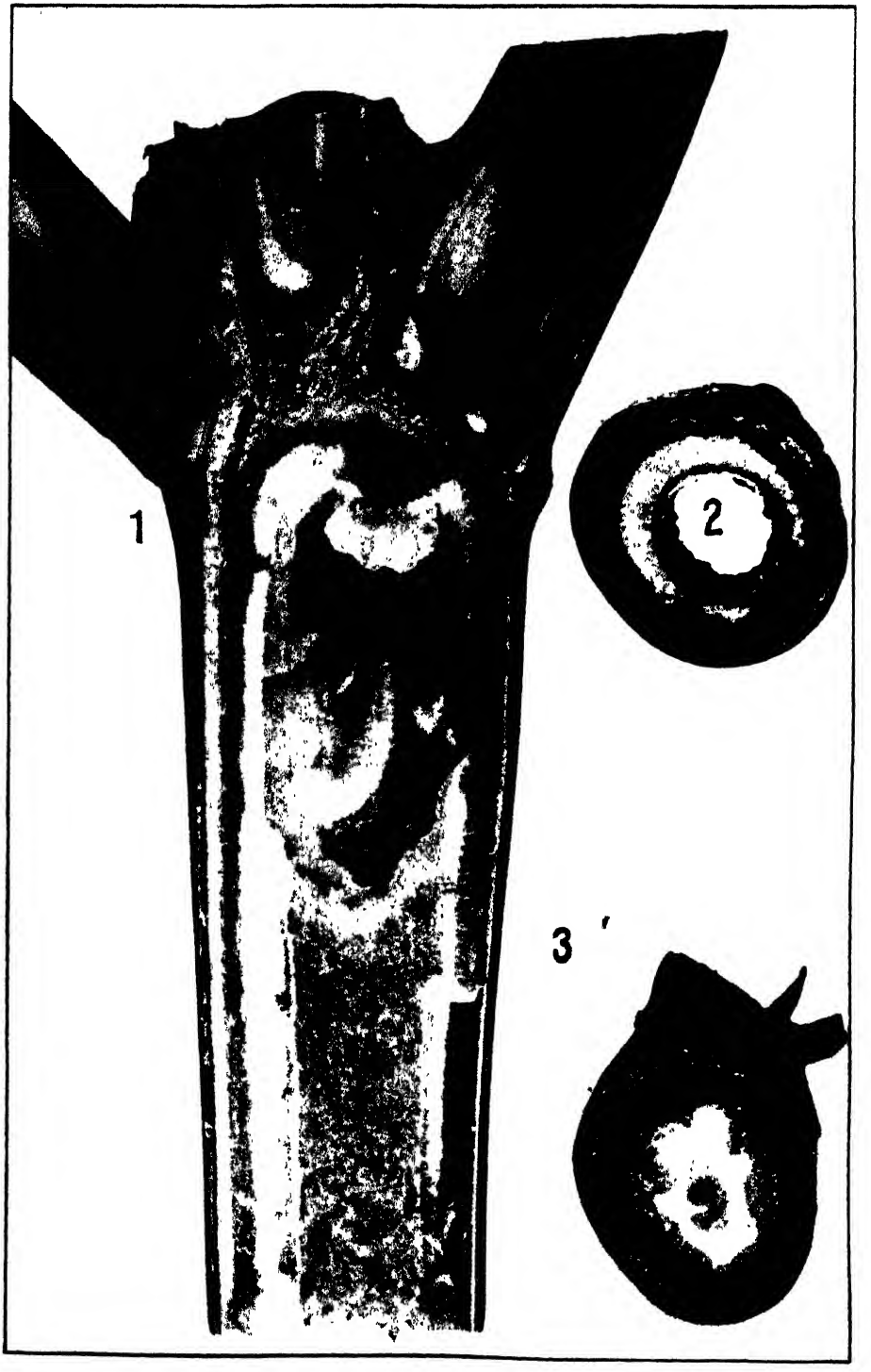
PLATE 14

1.—Longitudinal section of a stem of *Riscnus communis*, showing copious proliferations due to a 5 per cent grape-sugar solution and restricted to the injected internode. Entire wall covered with granulations in the lower part of the figure. The large nodule below the black-paper background developed around the needle entrance. The white band behind this at the left and on the lower right side is dead pith. ×2.

2.—Cross section below figure 1. The dead pith (white ring) encircles the inner, still living granulations. Injected August 7, 1916. Photographed on August 24. ×2.

3.—Cross section of a Ricinus internode which received 0.2 c. c. of clear lime water when young. No evidence of wound-repair tissue, but the pith cavity filled solid (save for a black speck in the center) with pith cells as in *a very young* internode. The white area represents the line along which the outer pith would later have separated from the central core. There was much diffuse red stain in the living pith outside of the white (dead) area, and this pigment which was also abundant in the internodes injected with sodium bicarbonate stained Carnoy's fluid decidedly red. This red stain is perhaps to be accounted for by an excessive movement of sugar into the treated internodes. The normal diameter of the pith cavity of a Ricinus stem of this age and shape would be about 17 by 12 mm. Internode injected on August 8, 1916. Photographed on August 24 about 1 inch above the lower node. Only the lower 2.5 cm. of the lumen (part below the needle entrance) was completely filled with tissue. ×1½ (nearly).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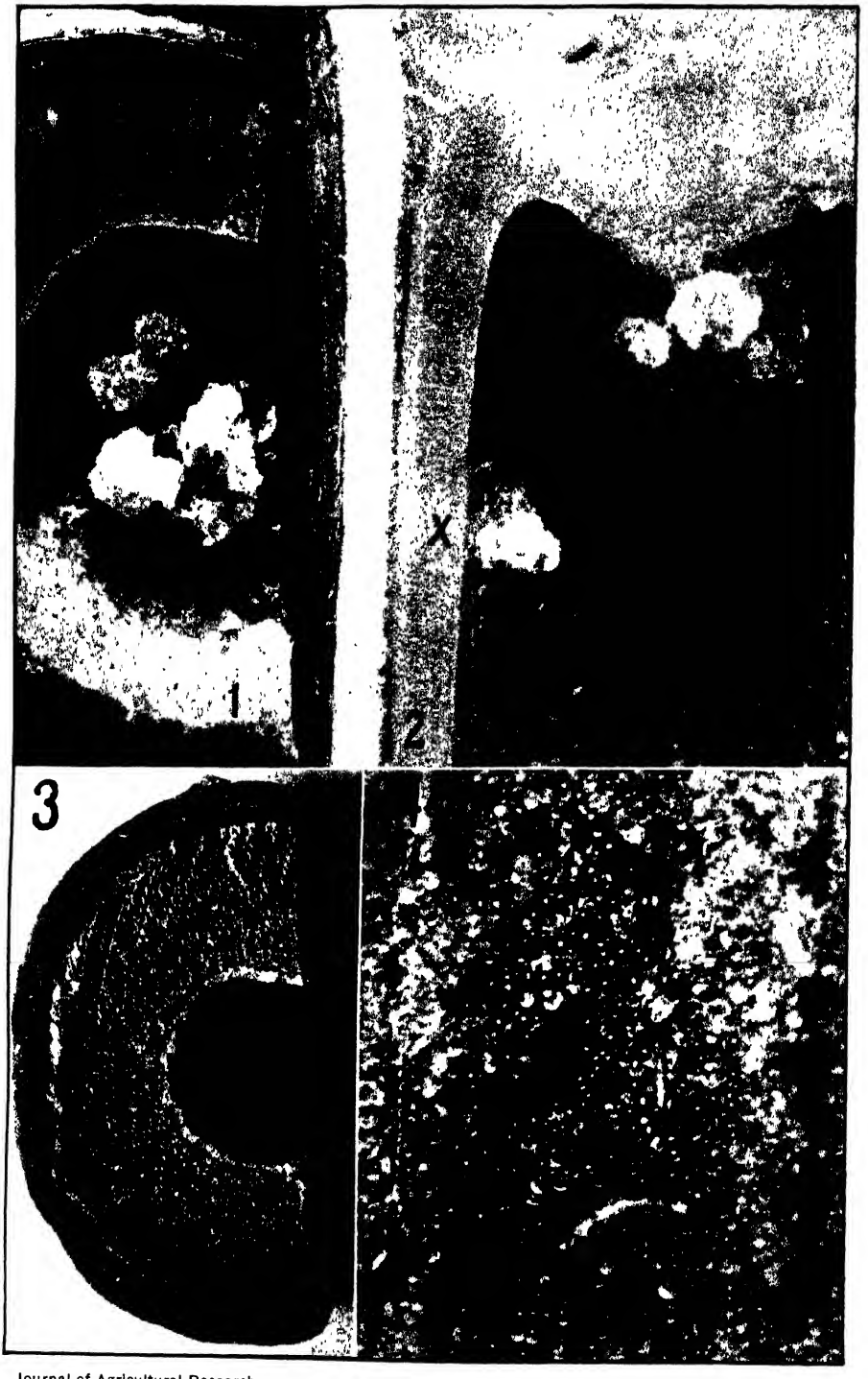
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PLATE 15

1, 2.—Internodes of *Ricinus communis*, showing the result of injecting a 5 per cent solution of cane sugar into young internodes of the main axis, the stimulus being confined to the injected internodes. (1) Ceiling of lower injected internode and (2) ceiling and upper part of middle injected internode, both showing conspicuous proliferations—that is, numerous small tumors. That at *X* is where the needle entered. There is no visible killing of the pith surface in the upper part of either internode. Injected on August 7, 1916. Photographed on October 19, 1916. ×3.

3.—Cross section of upper part of a young Ricinus stem showing pith cavity lined by a thin sheet of glistening white cell proliferations due to the injection of ammonium tartrate. Time, nine days. ×10 (circa).

4.—Side view of figure 3, further enlarged to show the cells.

PLATE 16

1.—Eight small tumors and various strands in an unopened internode of *Ricinus communis*, which showed no surface injury of the pith. On the opposite side of the pith cavity facing these nodules was a continuous white tumor an inch long. This is the first internode above one into which was inserted a small sterile glass tube holding 0.2 c. c. of a 5 per cent solution of monobasic ammonium phosphate, which slowly gives off alkaline vapors, and between the two internodes there was ¾ inch of solid node tissue. Tube inserted on August 8, 1916. Photographed on August 30. ×5.

2.—Section of one of the tumors shown in figure 1. Four unopened internodes in this stem showed effect of the vapor—namely, the first one below that which held the tube and the three immediately above it.

2
1

PLATE 17

1, 2.—Cross sections of two internodes of *Ricinus communis* (two plants), showing pith proliferations due to the feeble alkaline vapors from monobasic ammonium phosphate. An open glass tube containing 0.2 c. c. of a 5 per cent water solution of the phosphate was inserted into the lower end of these internodes and sealed in on August 7, 1916. The plate shows sections of the upper end of each internode. The lower was more responsive than the upper, probably because younger, although the upper shows granulations from all parts of the pith surface. Photographed on August 17. ×8.

PLATE 18

1.—Top of lower injected internode of plant 1 of *Ricinus communis*, which on August 15, 1916, received 1 to 100 ammonia water (one part aqua ammonia sp. gr. 0.90; 100 parts distilled water) and which shows numerous proliferations from the ceiling.

2.—Section 1 inch below figure 1, but above the needle entrance. This shows proliferations from the side wall with no dark specks on the pith. Photographed on October 3, 1916. ×5.

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PLATE 19

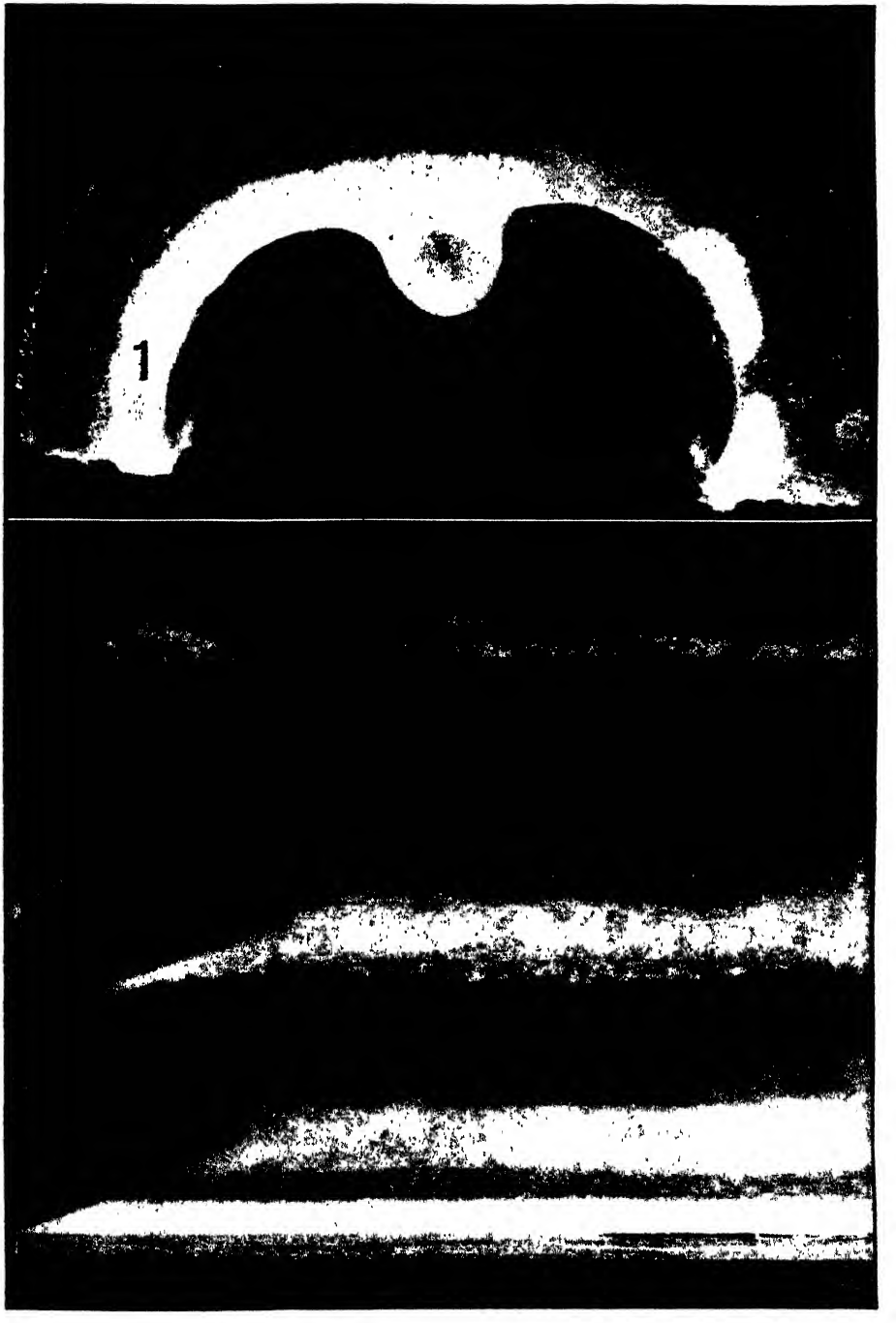
1.—Floor of upper injected internode of the main axis of plant 1 of *Ricinus communis*, showing copious proliferations from the pith. This was injected with 1 to 100 ammonia water on August 15. Photographed on October 3, 1916. ×4.

2.—Same as figure 1, but with the stem pared down one-fourth inch to remove shadows and show the proliferations in their proper color (pure white). Photographed on October 3, 1916. ×4.

PLATE 20

1.—Plant 1 of *Ricinus communis*, which received 1 to 100 ammonia water. Cross section of first (untreated) internode above the one figured on Plate 19, showing on one side an excess of pith, in the middle of which are displaced vascular bundles. There are no surface granulations. ×5.

2.—Side view of the same. The ridge extended the whole length of the 6-inch internode, through the floor of which there was an abnormal opening into the treated internode below. Vascular strands at *X*. Injected August 15, 1916. Photographed on October 3. ×5. For the structure of the strands see Plate 43.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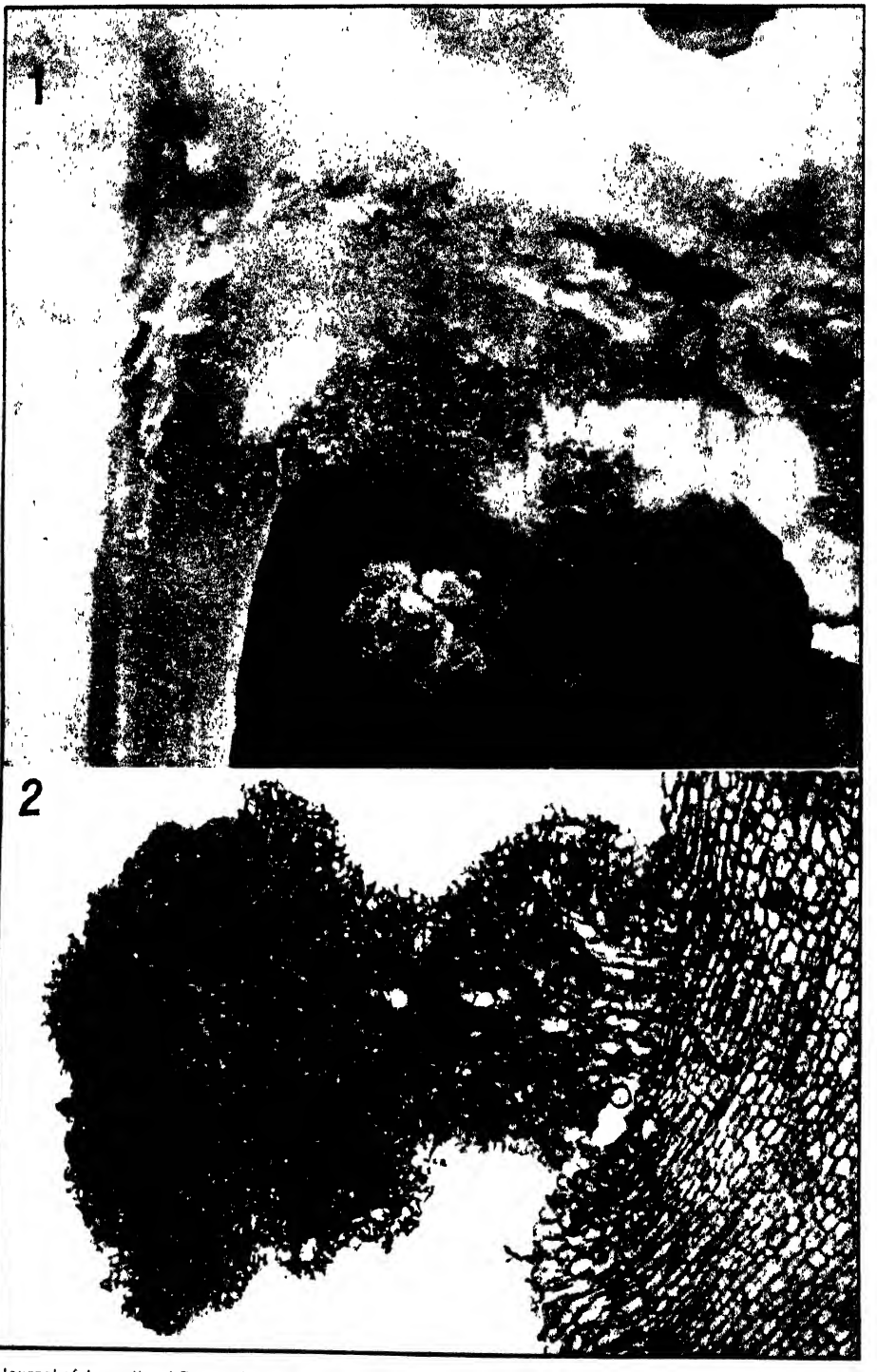
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PLATE 21

1.—Roof of second unopened internode below lowest injected one on main axis of plant 1 of *Ricinus communis*, which was injected with 1 to 100 ammonia water on August 15. This node gave rise to a branch the lowest internode of which had been injected. At the extreme top is shown the floor of the first unopened internode below the lowest injected one on the main axis, and here there are no proliferations, but there was a growth on the ceiling above this floor. Photographed on October 3, 1916. ×5.

2.—Free-hand unstained section of tumor shown in figure 1. The section is too thick; it remained overnight in water, and it is not sufficiently magnified. The right-hand part is normal tissue. Planar f. 35 mm., bellows 55.

PLATE 22

1, 2.—Plant 5 of *Ricinus communis*, injected August 15, 1916, with 1 to 1,200 ammonia water (aqua ammonia sp. gr. 0.90, 1 part, distilled water 1,200 parts). Both faces of the upper part of the second injected internode (main axis) show knobs and glistening strands of proliferated pith tissue. Below there was a continuous sheet of glistening proliferated cells. There were evidences of pith injury in the lower pat of the internode but none in this part. Photographed on September 11. ×3.

y

PLATE 23

Plant 5 of *Ricinus communis*, branch A, showing effect of 1 to 1,200 ammonia water. These are sections of the fourth (X), third, second, and first internodes of the uppermost untreated branch.

All show white nodular proliferations, and Y also shows strands; none were injected; and none show pith injury (dark specking). This branch was very young or not yet developed at the time of the inoculation, but it grew out of that node of the main axis which formed the ceiling of the uppermost injected internode (see Pl. 27, fig. 3). Somewhat below the surface, in the shadow, on the right side of the pith wall of X, are also half a dozen small tumors. Plant injected August 15, 1916. Photographed on September 11. $\times 3$.

PLATE 24

1.—Plant 5 of *Ricinus communis*, branch B, injected August 15, 1916, with 1 to 1,200 ammonia water, showing proliferations from the ceiling and side walls of the second internode above the uppermost injected one. There are half a dozen side-wall tumors, as well as the central one. Surface of the pith uninjured. ×5.

2.—Section of small marginal proliferation on wall of pith cavity shown in figure 1. ×160. Compare with Plate 29, figure 6.

3.—Same as figure 1, but from branch C. This is the third internode above the uppermost injected one. There were also proliferations in the first and second internodes above the injected one. Here the proliferations are all on one side in a perpendicular line, all are inconspicuous elevations, and the pith surface as a whole is entirely normal. Photographed on September 11, 1916. ×3.

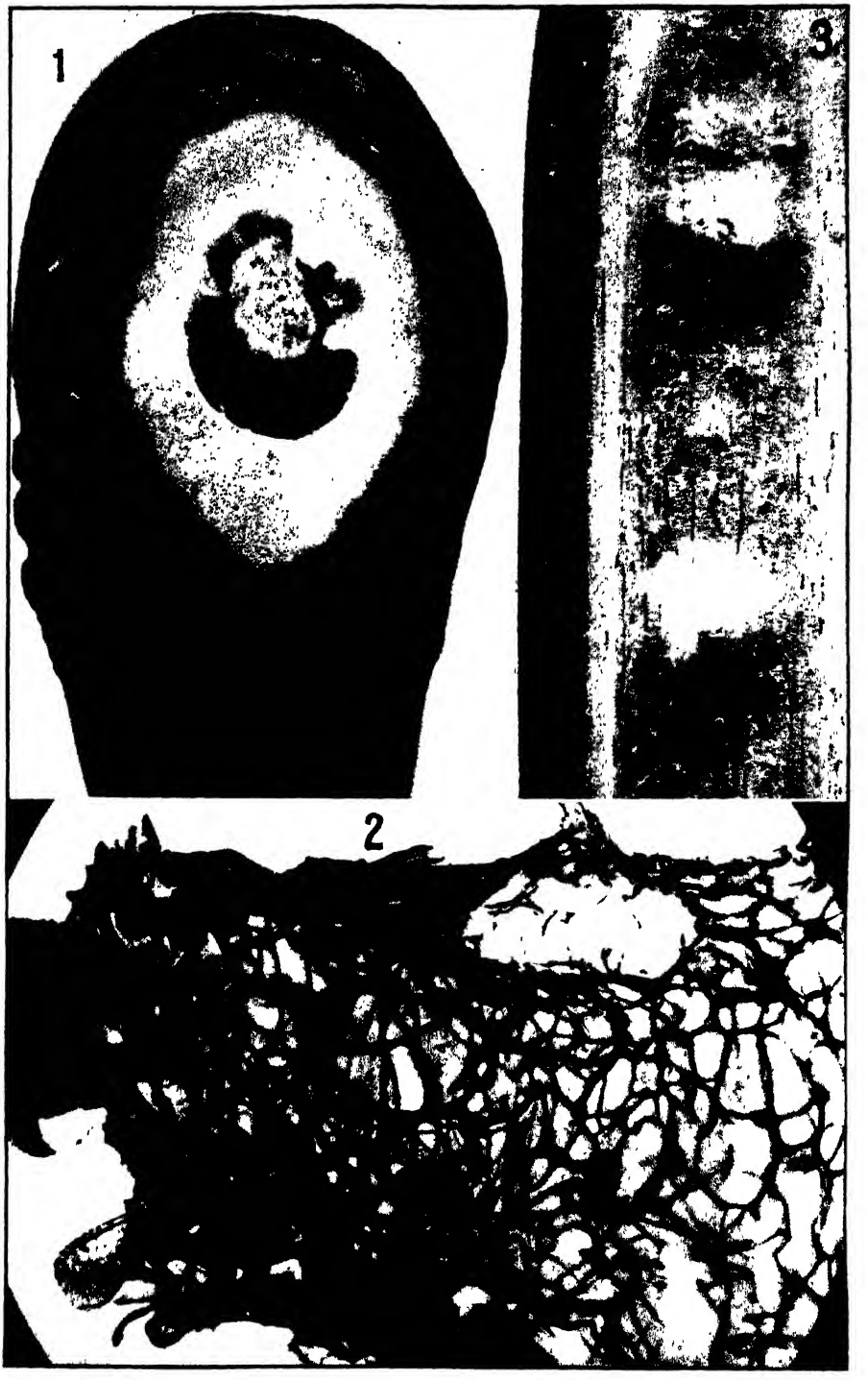
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PLATE 25

Upper half of first internode above the upper injected one in the main axis of plant 5 of *Ricinus communis*, which was injected August 15, 1916, with 1 to 1,200 ammonia water. The wall is thickened and there are numerous small proliferations—stringy growths and nodules. The surface pith between is normal, but the deeper layers of the pith are white (occupied by air). Photographed on September 11. ×5.

PLATE 26

Lower half of same internode as Plate 25, showing continuation of tumors and strands. ×5.

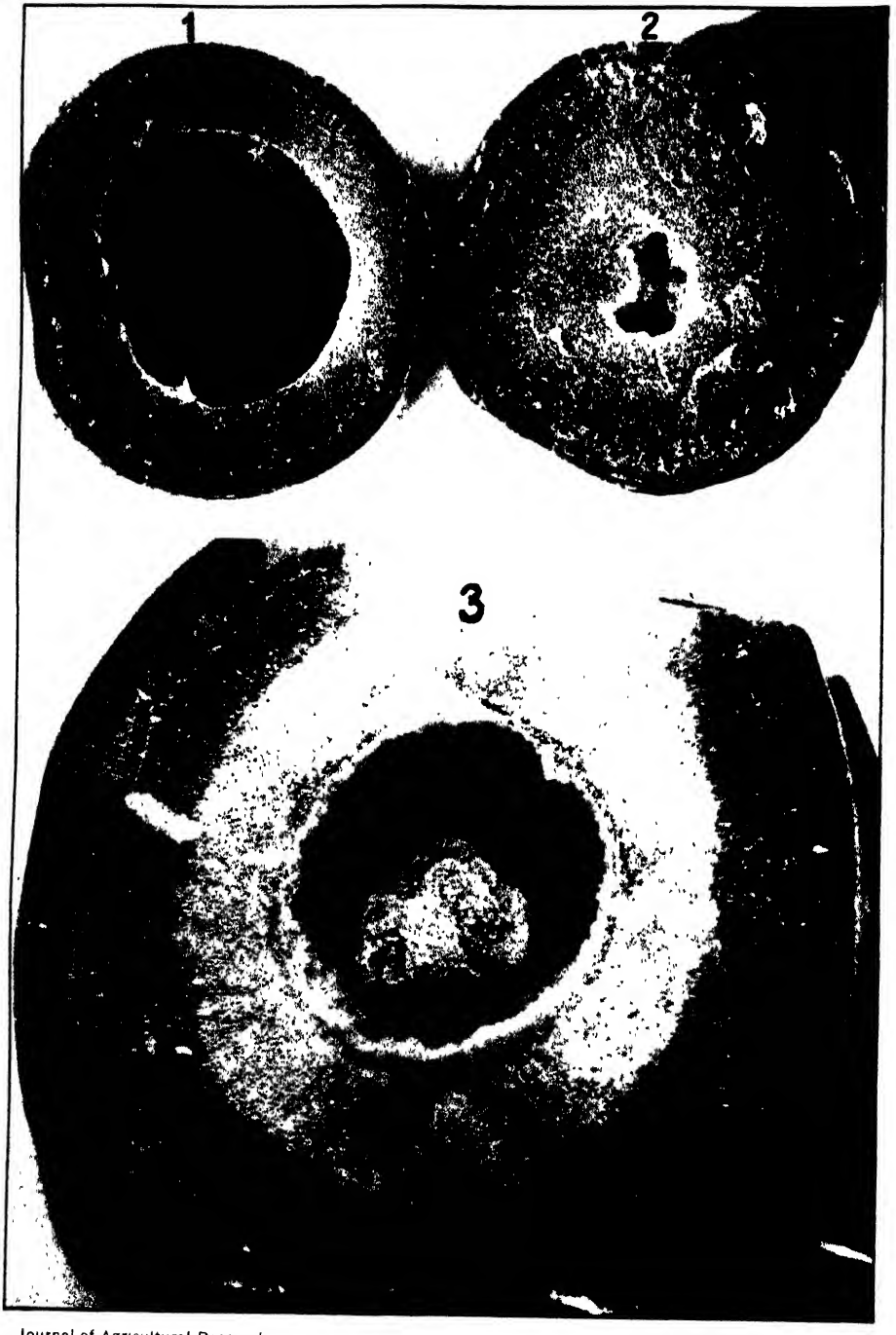
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PLATE 27

1.—Cross section of a normal internode of the same age as figure 2.

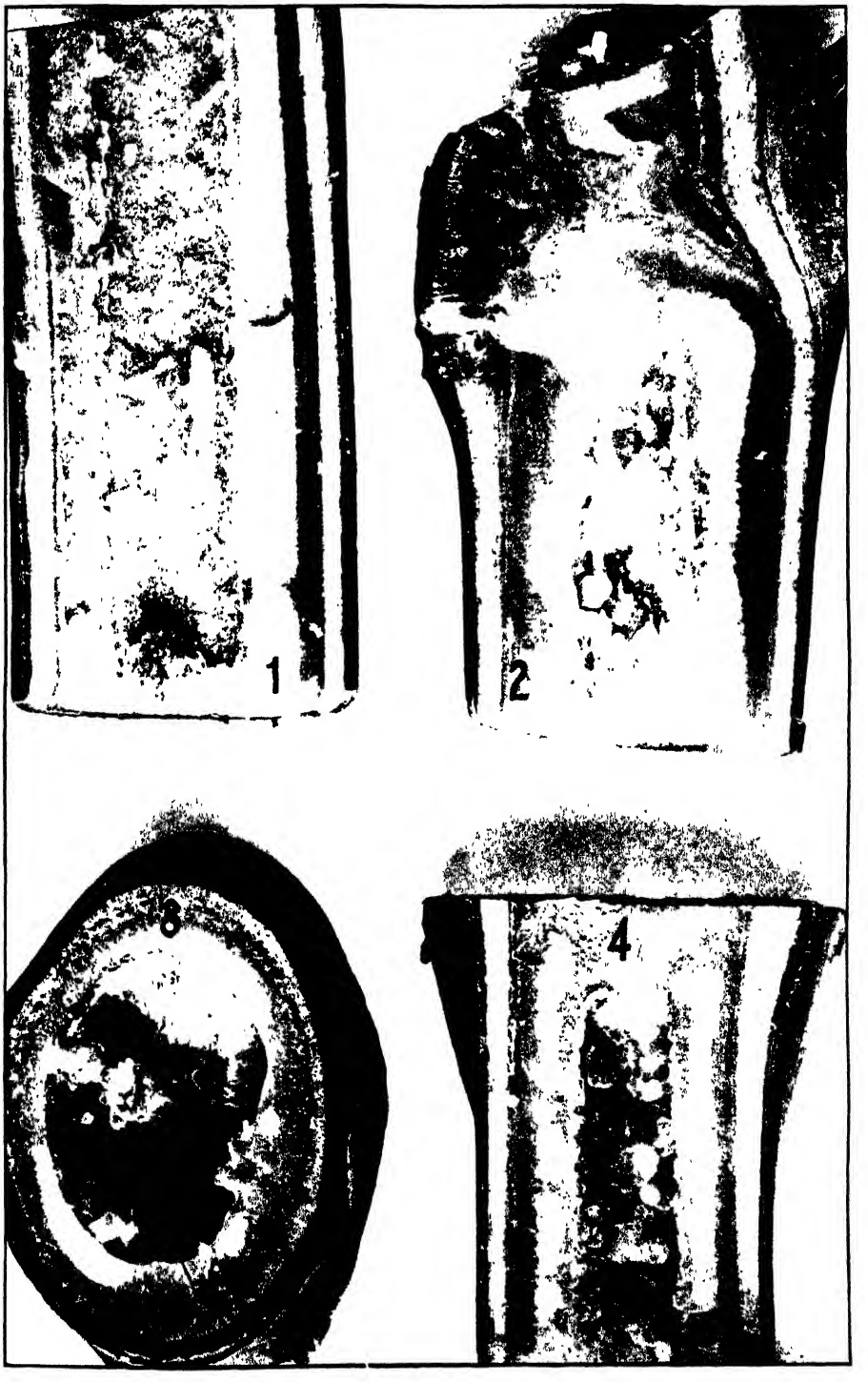
2.—Main axis of plant 5 of *Ricinus communis*, injected August 15, 1916, with 1 to 1,200 ammonia water. Cross section of fourth internode above the uppermost injected one showing a much thickened wall and nodular proliferations from opposite sides of the inner pith, which did not show any dead cells. Every one of the four internodes above the uppermost injected one showed striking proliferations from the side walls and floors and no specking of the pith—that is, no surface injury. Photographed on September 11. ×4.

3.—Main axis of Ricinus plant 5, injected with 1-to-1,200 ammonia water, showing nodular proliferations from the ceiling and thickened sides of the uppermost injected internode. There was also a sheet of proliferation 1 mm. thick over the whole inner surface of the internode, and on this there were about 50 protuberant small tumors (glistening white cell masses). There was no dark specking of the pith, but the deeper layers were white—that is, permeated by air. The floor also bore nodules like the ceiling. Photographed on September 11. ×5.

PLATE 28

1, 2, 3, 4.—Plant 4 of *Ricinus communis*, injected August 15, 1916, with 1 to 1,200 ammonia water. Various portions of injected internodes all the proliferations of which are from the inner pith. In figure 1 there is a continuous sheet of granulations bearing nodules and strands.

Figure 3 is a ceiling. Photographed on September 11, 1916. ×2.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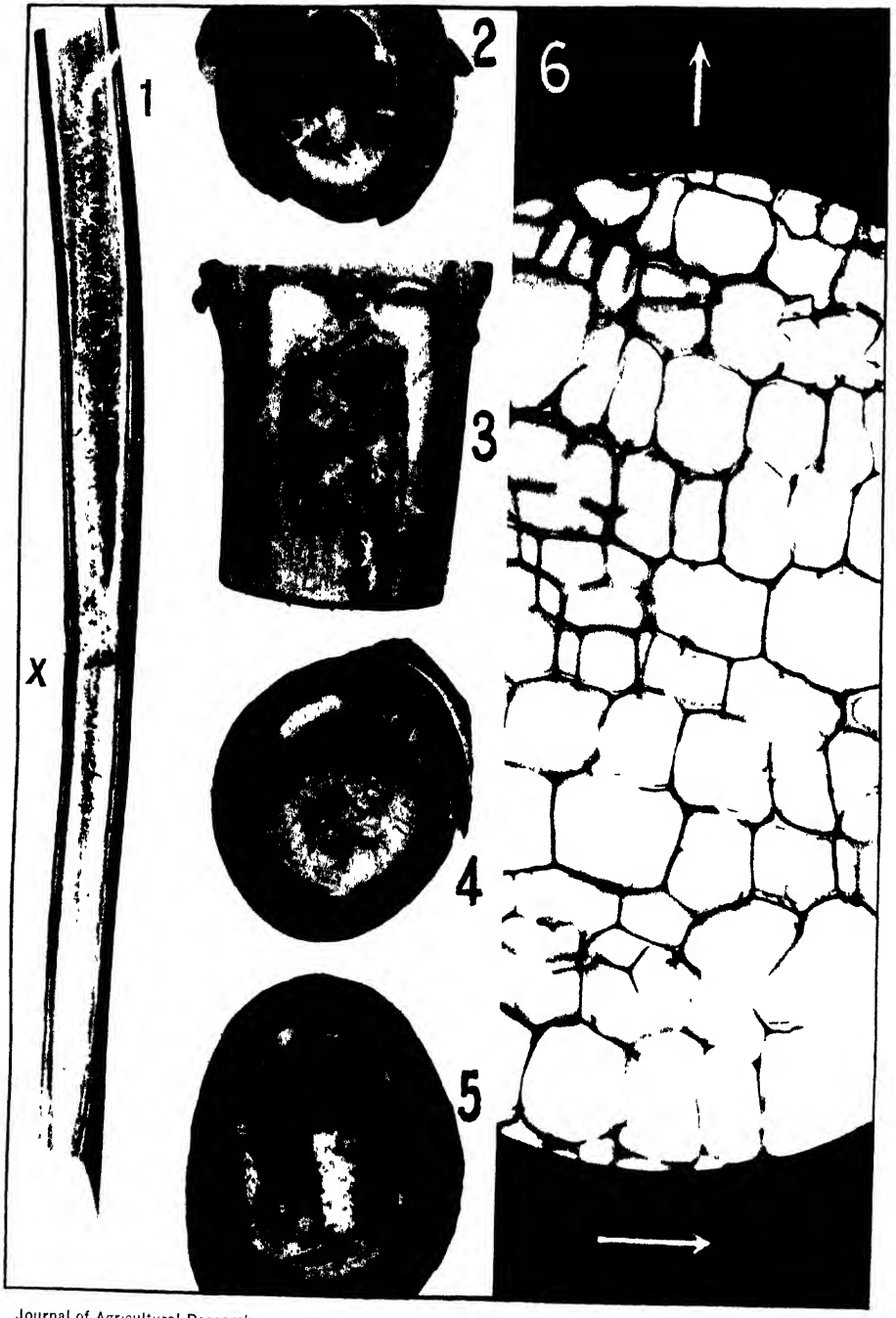
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PLATE 29

1–5.—Petiole and stem sections of plant 4 of *Ricinus communis*, which was injected on August 15 with 1-to-1,200 ammonia water.

1.—Middle of petiole, needle entrance at X. Pith much thickened (proliferated) and torn away in the upper part, leaving a rough-coated hollow cylinder of proliferated tissue. (See also Pl. 37 and 42.)

2, 3, 4, 5.—Parts of four unopened internodes at the top of the plant—that is, remote from the injected internodes, showing white glistening nodules in tissues which developed mostly after the injection. One of these sections is from a branch and the others are from the main axis. Between the uppermost injected internode and these nodules two and three nodes intervened Photographed on September 11, 1916. Petiole natural size. Figures 2 to 5, × 2.

6.—Normal pith from Plate 24, figure 1. It joins on (in direction of the upper arrow) to right side of Plate 24, figure 2. Arrow at bottom indicates the longer axis of the stem. ×160.

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PLATE 30

Ceiling of upper treated internode of plant 2 of *Ricinus communis*, which was injected with 1-to-1,200 ammonia water on August 15, 1916. It shows lobed tumors from the center and also from a dozen places on the side walls. There was here no trace of killing action (surface specking) of the pith. Photographed on September 9, 1916. ×5.

PLATE 31

Ceiling of second (middle) injected internode of plant 3 of *Ricinus communis*, which received 1-to-1,200 ammonia water on August 15, 1916. It shows granulations and nodules and no killing of the tissues. The dark marginal stripes on the cavity wall are normal red stripes due to superposed pigmented pith cells which also occur freely in the deeper parts of the normal pith of *Ricinus communis*. Photographed on September 9. ×5.

PLATE 32

1, 2.—Stem of *Ricinus communis* exposed to the vapors of monobasic ammonium phosphate (20 per cent in water). Figure 1 is a front view and figure 2 a side view of nodular growths which developed on the ceiling of the unopened internode next below the one into which was sealed a sterile glass tube containing 0.2 c. c. of the fluid. Experiment begun August 21, 1916. Photographed on September 2. ×5.

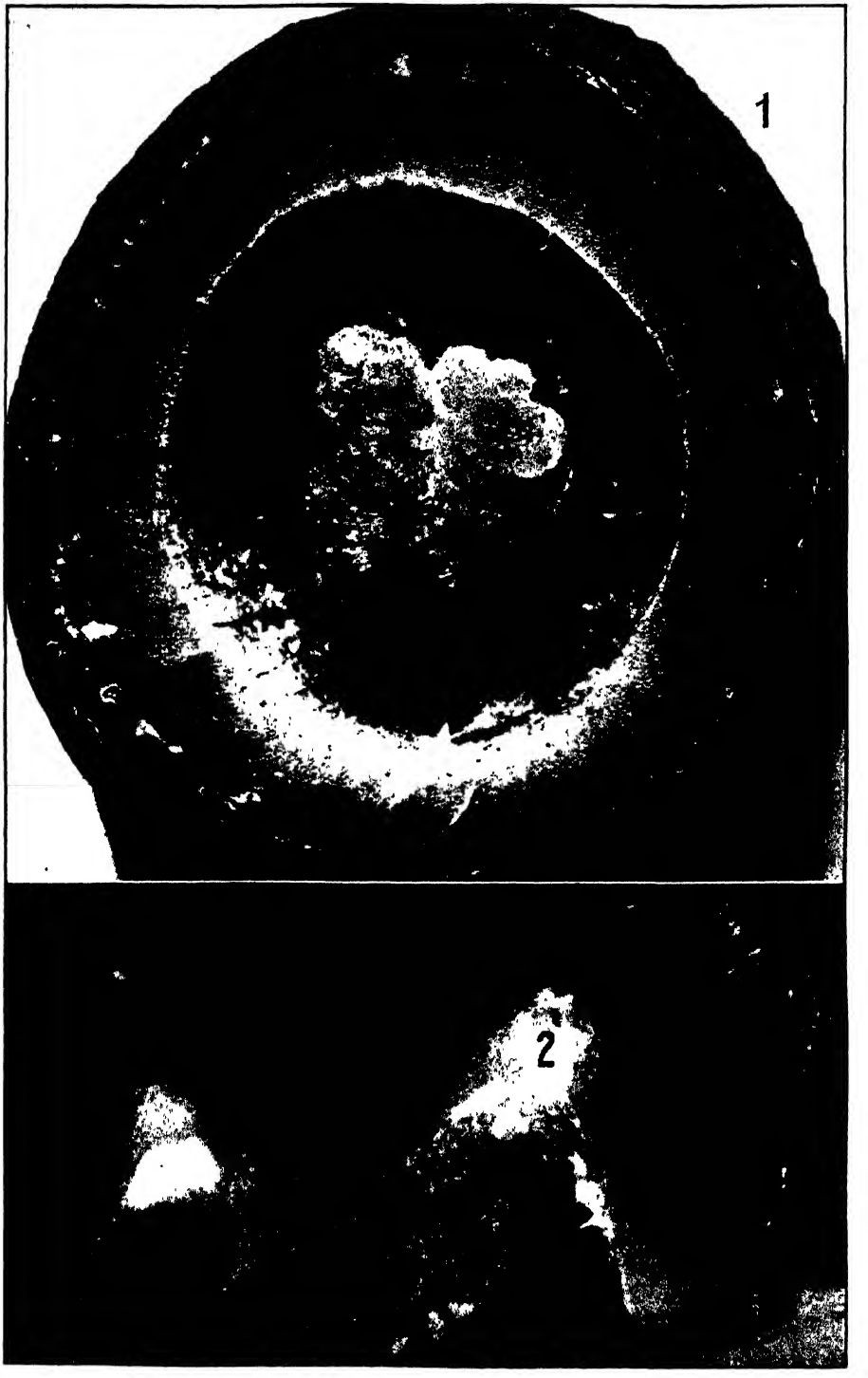
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PLATE 33

Stem of *Ricinus communis* in cross section showing pith-lining of a normal node for comparison with Plates 32, 34, and others. The middle of the node wall often bears a smooth, cone-shaped projection as at *X*. Photographed on September 5, 1916. ×8.

PLATE 34

Longitudinal section of stem of *Ricinus communis* showing further effects of vapor of monobasic ammonium phosphate. Same plant as shown on Plate 32, but in another place—that is, in the upper end of the tube internode showing ceiling and side-wall tumors, and the lower end of the unopened internode above bearing a floor tumor, the tip of which (*X*) is normal (see Pl. 33). Exposure begun on August 21, 1916. Photographed on September 2. ×5. For a longitudinal section through a normal node of the same age see Plate 35.

X

VB
X

PLATE 35

Longitudinal section through a normal node and part of the two adjoining internodes of a well-developed plant of *Ricinus communis*. *B*, Bark; *Xy*, wood; *P*, pith. Bundles of anastomosing vessels (*VB*) are visible in the middle of the node as white bands. ×5.

PLATE 36

Top of an unopened internode in a stem of *Ricinus communis* next under one that had sealed into it a tube containing 0. 2 c. c. of a 20 per cent solution of monobasic ammonium phosphate. It shows a thickened cone and nodular proliferations from its apex. The white patches in the pith are dead cells and below the surface here shown they are united. In cross section at the extreme left are the vascular bundles of the node showing as dark spots (*VB*) and above and below as strands. Experiment begun August 21, 1916. Photographed on September 5. ×5.

VP

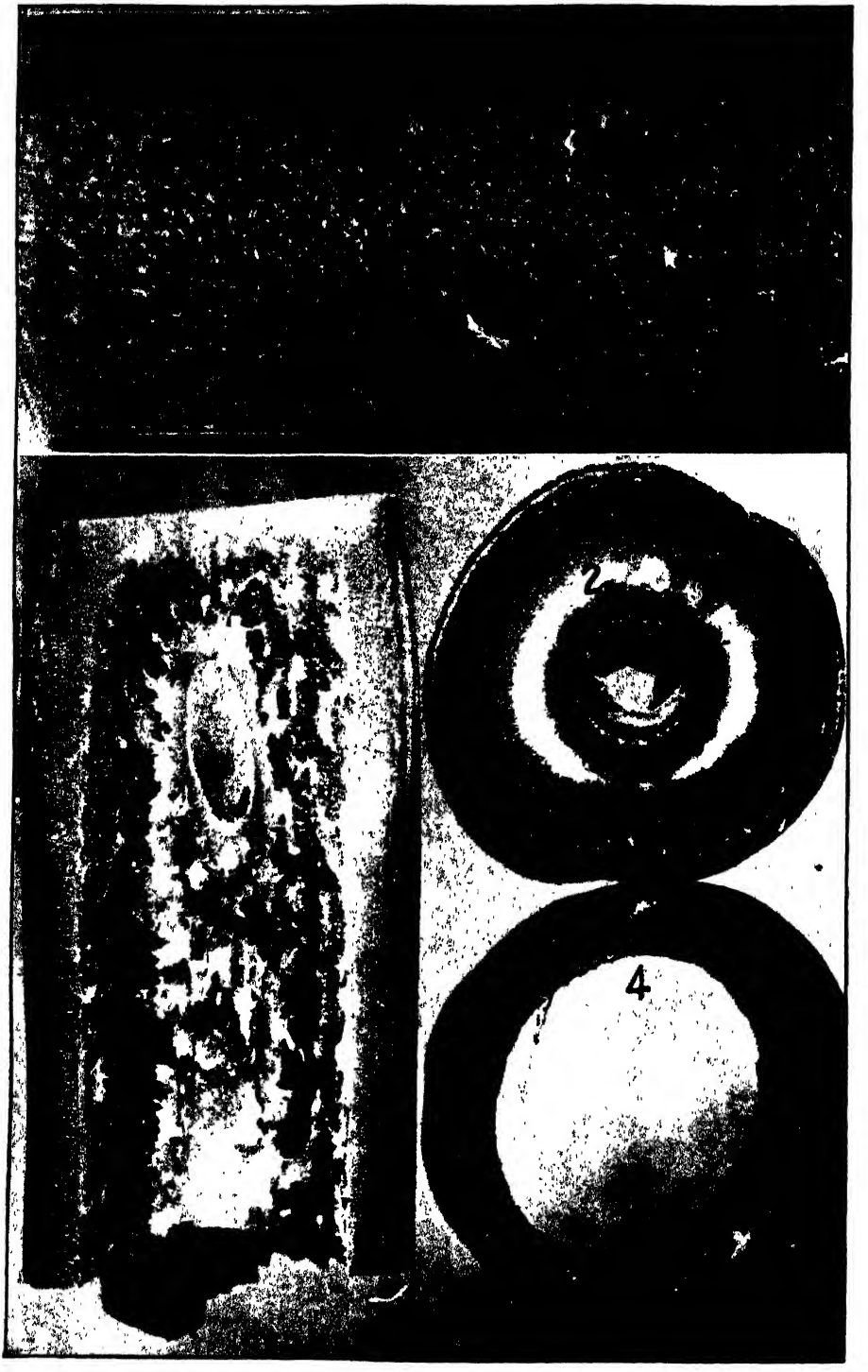
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PLATE 37

1.—Longitudinal section of a normal young internode of *Ricinus communis*. The white specks are shriveled (dead) pith fragments dating from the formation of the pith cavity. Photographed on July 27, 1916. ×4.

2.—Cross section of a Ricinus internode into which on July 22 was injected a 5 per cent solution of monobasic ammonium phosphate This caused a great thickening of the pith with a continuous sheet of surface granulations behind which a second xylem-phloem cylinder has developed and between which and the normal xylem-phloem cylinder is a wide area of pith, the middle part of which is losing water (becoming white) and preparing to form a cylindrical cavity At other levels this cavity was already visible. Time 12 days ×4.

3.—Injected internode of another plant in the same series. Here the proliferated pith, the inner part of which is still living, has split away entirely from the outer normal pith and lies as a rough-coated hollow cylinder loose in the pith cavity. The white flecks on its surface are torn masses of dead and shriveled pith cells. ×4.

4.—Cross section of a normal internode of the same age as that in figure 2, for comparison with the latter. Photographed on August 3, 1916. ×4.

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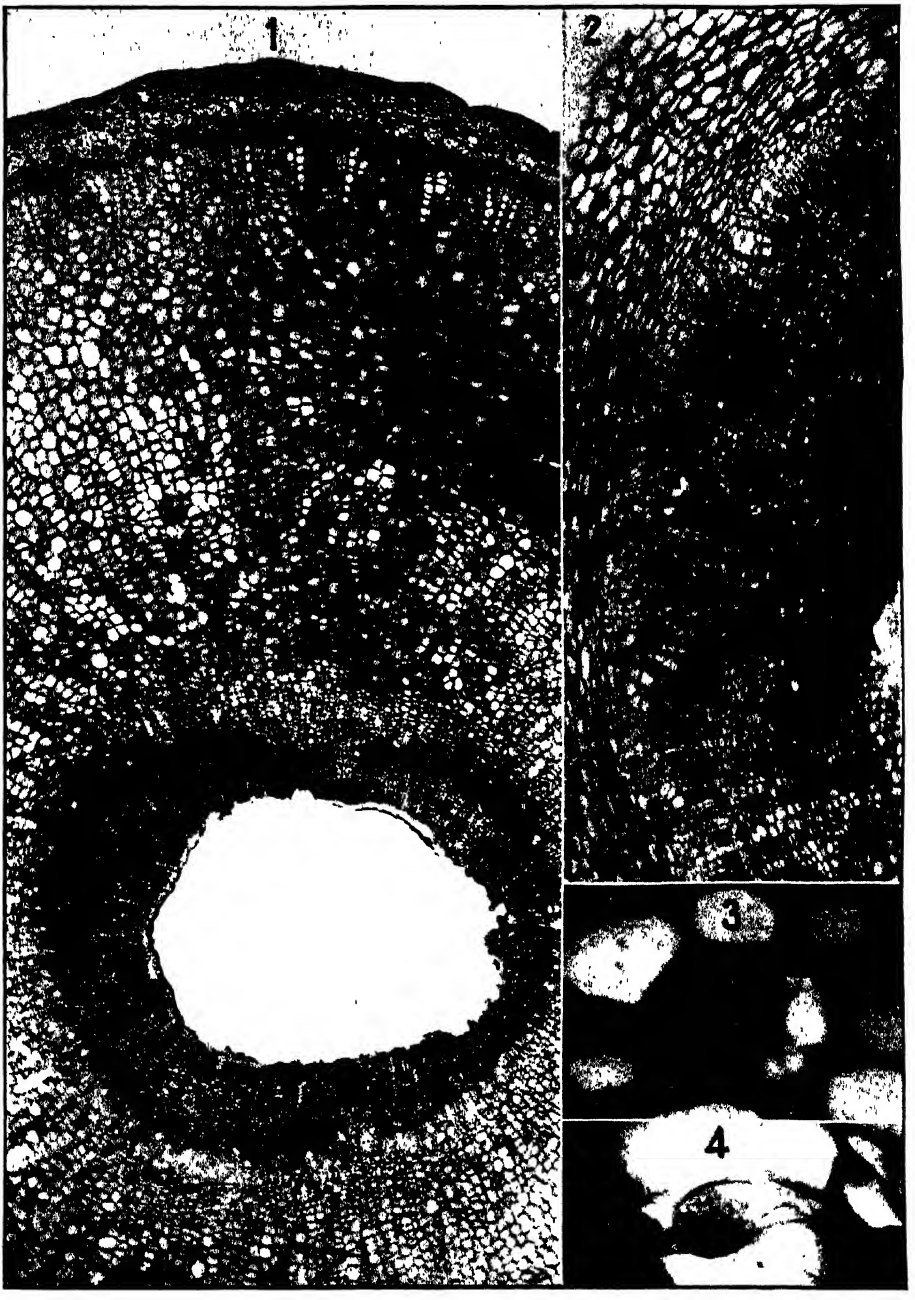
PLATE 38

1.—Same as Plate 37, figure 2, but from an embedded stained section. It can be seen that the pith cavity is irregular and that the second xylem-phloem ring is farther from the pith cavity on the left side than it is on the right side. It is also more irregular than the normal xylem phloem—that is, at top and bottom. The heavily stained cells in the pith are those which show white (dead) in Plate 37, figure 2. The interrupted dark line a little way in from the pith cavity is another cambium, not accompanied by vessels.

2.—A detail of the second xylem-phloem ring showing irregularities more distinctly. Planar enlargement.

3.—Sieve plate from second xylem-phloem ring. ×650.

4.—Distorted sieve tube from third (imperfect) xylem-phloem ring. ×650.


1
2
3
4

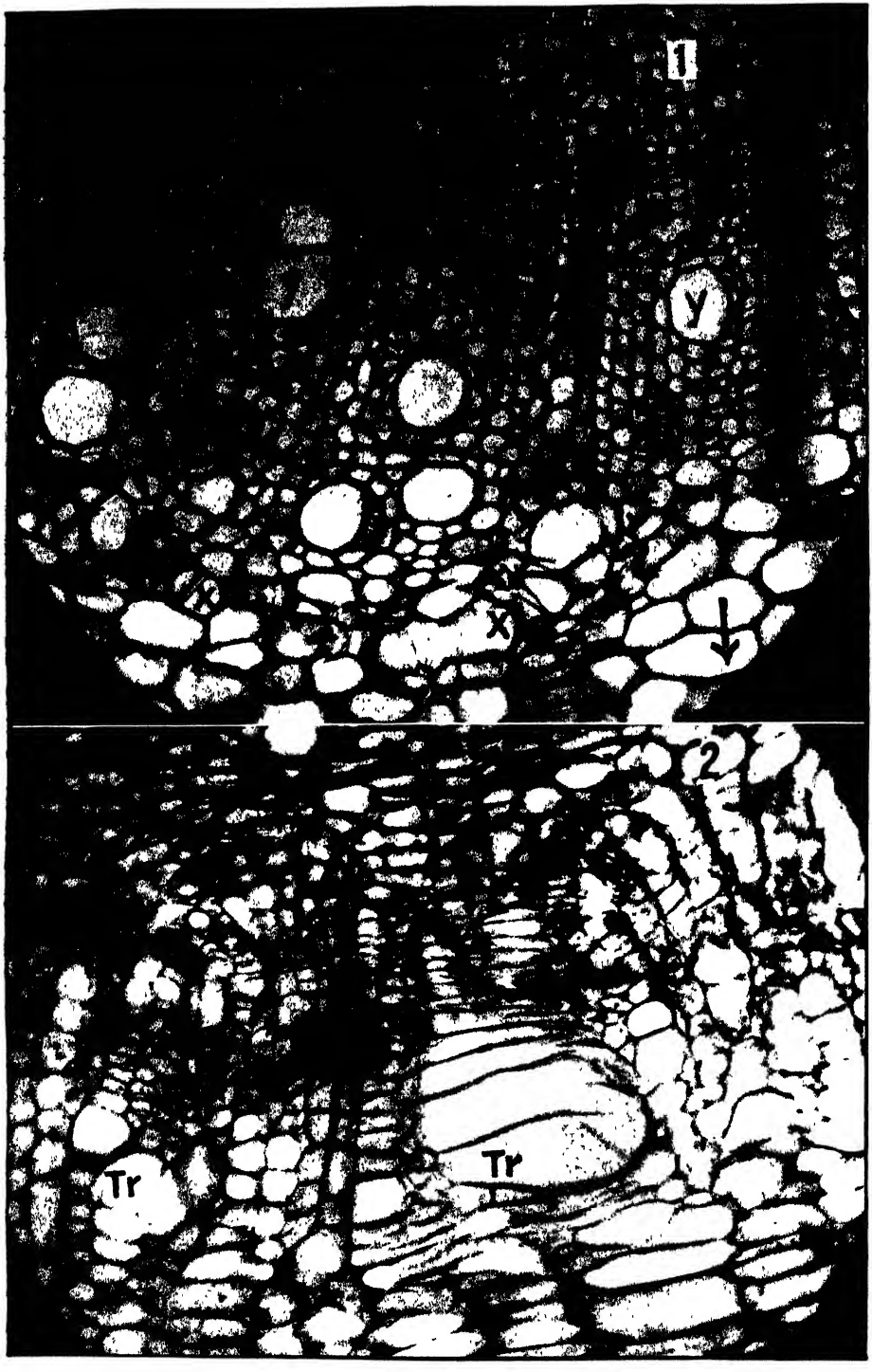

1
Y
X
2
Tr
Tr

PLATE 39

1.—Enlarged cross section of a rather regular portion of the second xylem-phloem ring in *Ricinus communis* (Pl. 37, fig. 2). The normal xylem-phloem ring is in the direction of the arrow. Cambium and phloem at the top. Twisted spiral vessels at *X*. Tracheæ properly oriented at *Y*. ×160.

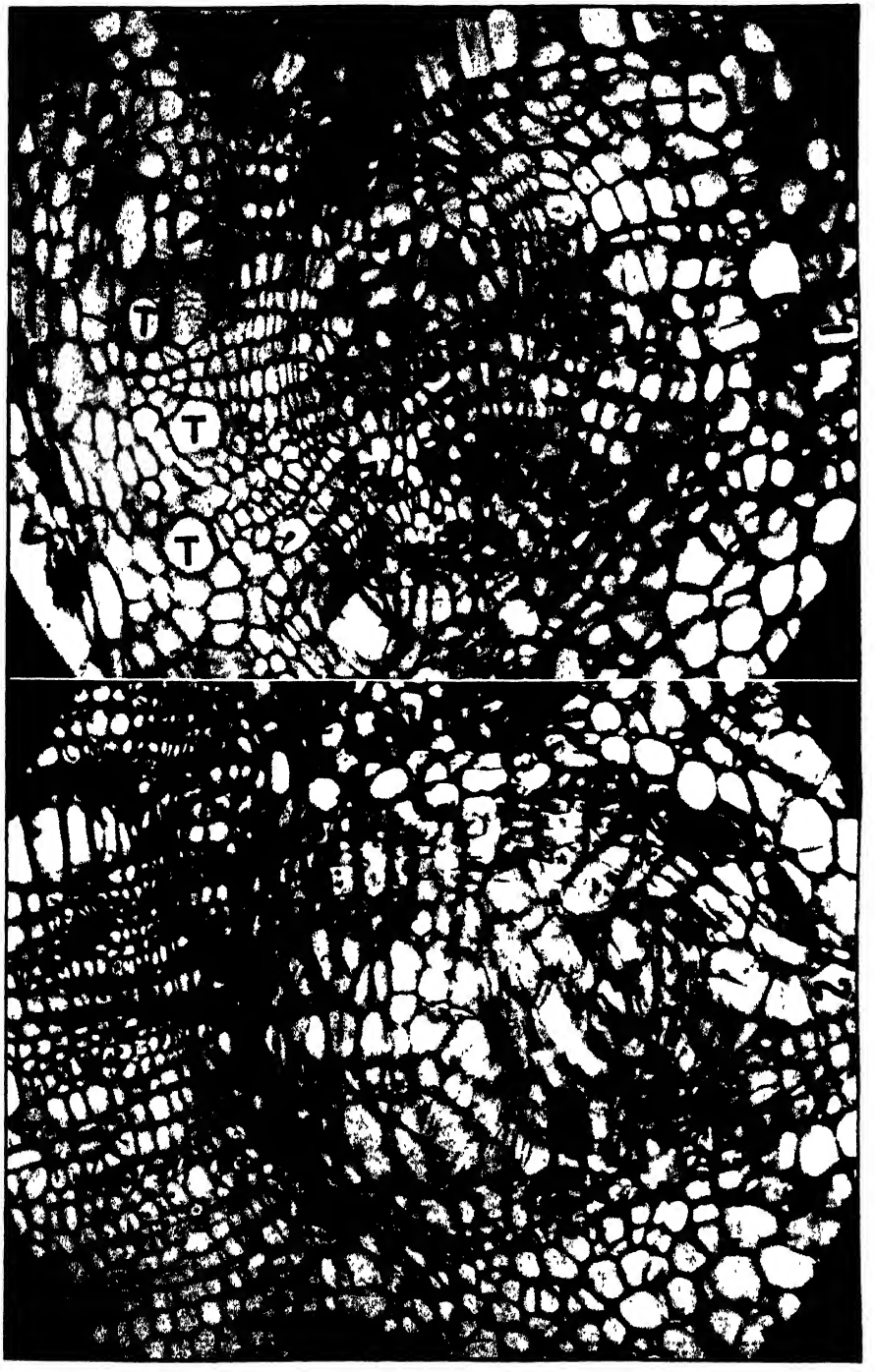
2.—From the same vascular ring, as figure 1, showing tracheæ lying at right angles to the longer axis of the stem, as often happens in crowngalls, and others properly oriented. Phloem at the top. Same magnification.

PLATE 40

1.—Cross section of second (pathological) xylem-phloem cylinder in Plate 37, figure 2, showing in the center a ring of cambial cells surrounding other cells (phloem). Below are tracheæ (*T*) some properly oriented, others lying at right angles to the long axis of the stem. The pith cavity lies in the direction of the arrow. ×160.

2.—Another swirl of cambium on the pith-cavity side of the second xylem-phloem ring in *Ricinus communis*. The xylem is in the lower part of the figure. ×160.

These cambial swirls appear to be concentric medullary bundles in the making Block 1265B.


T
T
T

Tr
Tr
Tr
2
Tr
3

PLATE 41

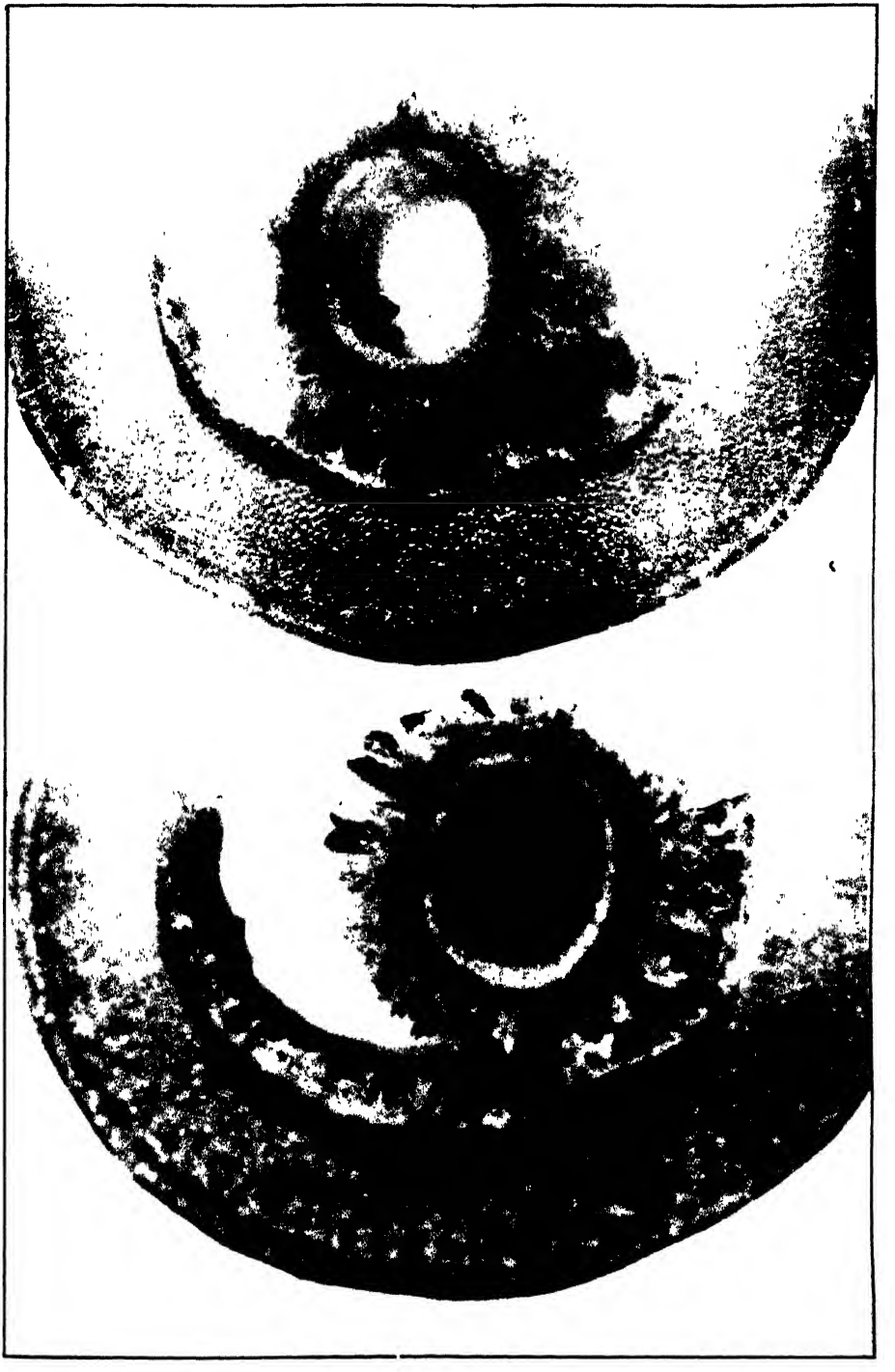
1. Second xylem-phloem ring in Plate 37, figure 2, showing a group of tracheæ, etc., lying at right angles to the long axis of the stem as in crowngall and sweeping out (direction of the arrow) into the pith (toward the pith cavity). Below, at either side, xylem-phloem normally oriented. *Tr*, tracheæ. ×160.

2. A portion of the fourth (imperfect) xylem-phloem ring in Plate 37, figure 2, showing tracheæ with cambium at their left, near the pith cavity, which is in the direction of the arrow, the second xylem-phloem being a little beyond this field at the right. ×160.

3. Like figure 2, but another part of the fourth xylem-phloem cylinder showing tracheæ, cambium, and sieve tubes. Same magnification. The pith cavity lies in the direction of the arrow.

PLATE 42

1, 2.—Cross sections of an internode of *Ricinus communis*, injected on July 22, 1916, (when young), with distilled water containing 5 per cent of dibasic ammonium phosphate. Much thickening of the pith, the inner pathological part of which has torn loose from the outer normal part. The cells in the region of the rupture were dead, but the remaining tissue of the separated pith cylinder, both the inner light ring of granulations and the middle dark ring, were fully alive. Photographed on August 3, 1916. ×8.



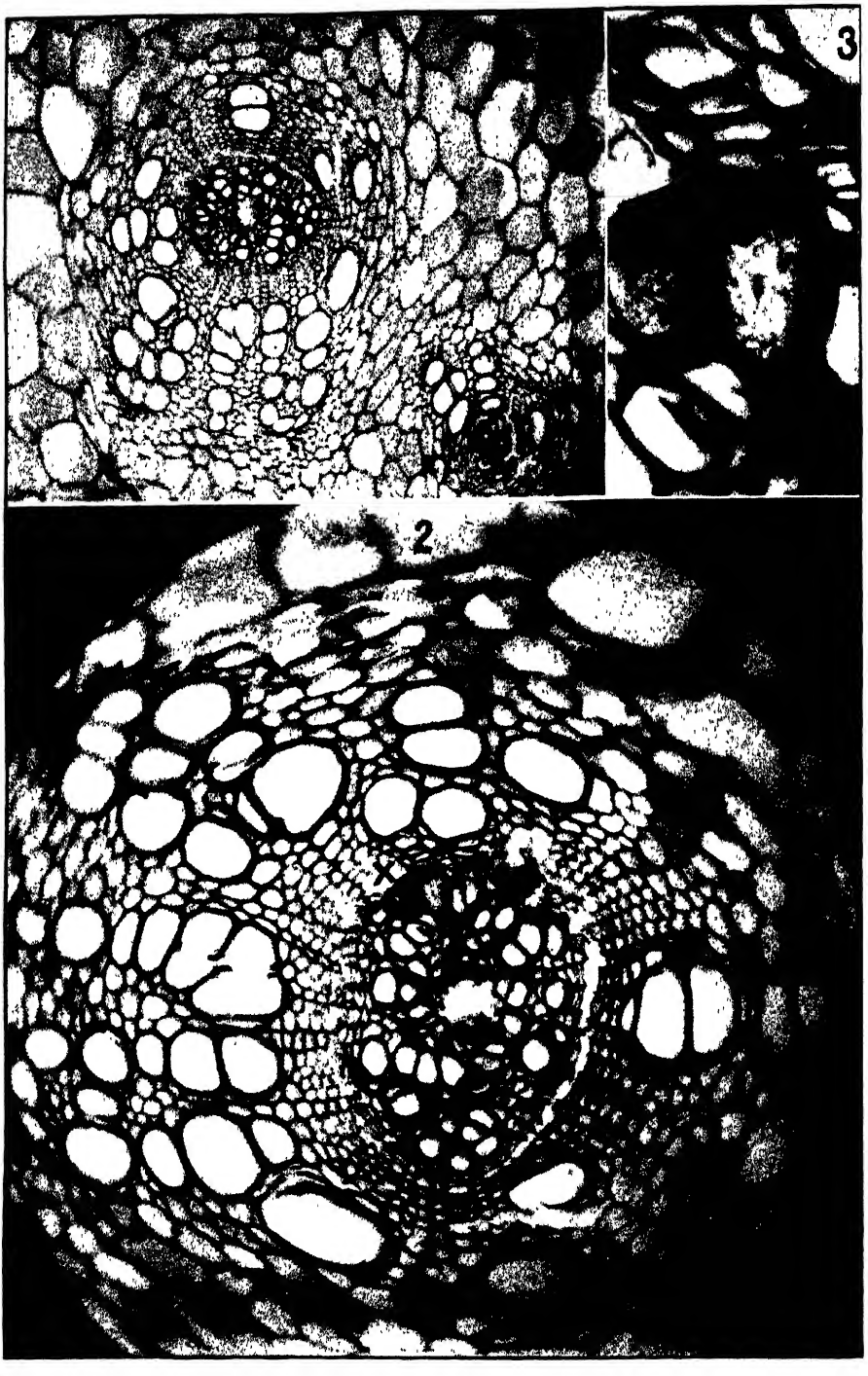

3
2

PLATE 43

1.—General view of the small displaced vascular strands (concentric medullary bundles) shown at *X* in Plate 20. The coarse-celled outer tissue is pith. ×75.

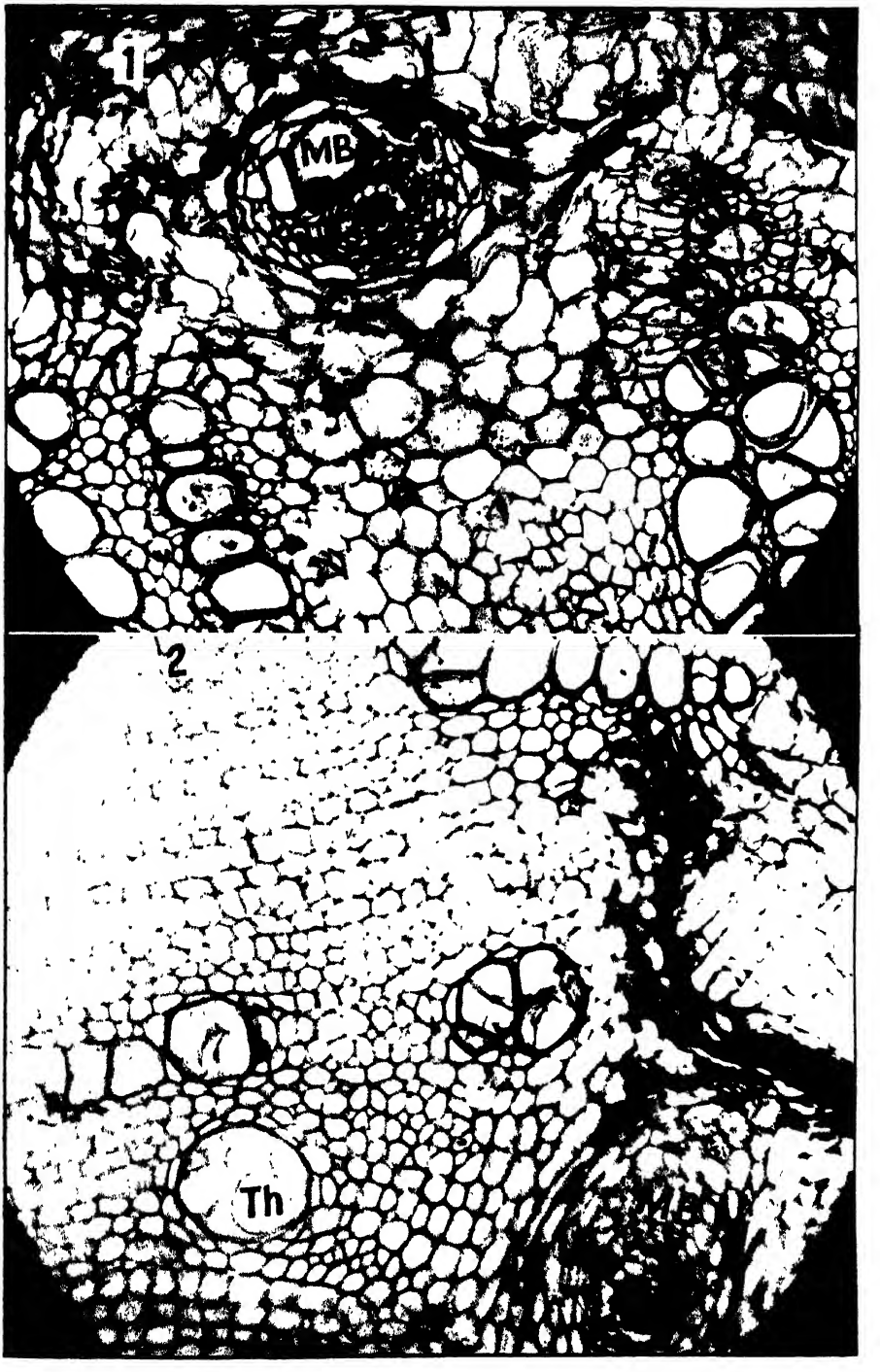
2.—Structure in cross section of the larger of the two strands in figure 1, xylem (spirals, tracheæ, etc.) on the periphery, cambium ring midway, phloem in the center. At *X* are two sieve plates. ×160.

3.—Sieve plates of figure 2 further enlarged. Block 1288. Slide 4. ×650. The perforations of these plates show quite well in the photograph, but are mostly lost in the overetched halftone.

PLATE 44

1.—Cross section of stem of *Ricinus communis* treated with ammonia (Pl. 5) showing the development of an abnormal (?) medullary bundle (*MB*) and its relation to the normal bundles. Pith above, inner part of woody ring below. ×160.

2.—Cross section of Ricinus stem treated with strong ammonia (Pl. 5) showing inner part of xylem, many tracheæ of which are filled with thyloses (*Th*) as in vascular bacterial diseases. At the right there is a little of the proliferated tissue, including one of the curious medullary bundles (*MB*) the center of which is phloem. Same magnification.


1
MB
2
Th

PP
VB
2

PLATE 45

1.—Inner three-quarters of one of the middle proliferations on Plate 5, showing its origin from the inner face of the vascular bundles (*VB*) with tracheæ and sieve tubes of its own at *Tr*. At *MB* are two concentric medullary bundles. × 70.

2.—Ricinus stem treated with strong ammonia (Pl. 5) showing four whorls of vascular tissue (concentric medullary bundles) similar to those occurring in crowngalls: i. e., the central tissue is phloem and the outermost cylinder is xylem. The pith cavity is in the direction of the arrow. Below is a branch gap (?). × 160. Compare with Plates 40, 43, and 65.

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PLATE 46

1, 2.—Cross sections of the killed tissue (pith) at one side of the proliferations shown on Plate 5. Above in each are rows of collapsed dead pith cells containing many crystals of calcium oxalate. Below are tissues which have proliferated from the inner face of the bundle sheath. Below at the left, in figure 1, bundle vessels are included. In figure 2, which joins on to the right of figure 1, the section cuts above the vessels of the bundles. In the middle of each section immediately under the killed (black) tissue (at *XX*) are vessels, and at *P* a little mass of tissue interpreted as new phloem, but no sieve plates were found in it. This phloem and the new xylem vessels must have formed in advance of the death of the tissues surrounding it (it was not itself dead tissue) and further growth stopped by the deeper killing in of the pith tissues. A similar small mass of phloem (also subtended by spiral vessels) is visible in the same relative position in sections made from the other side of the proliferations shown on Plate 5. This is, I believe, the interrupted beginning of a second xylem-phloem ring. ×160.

3.—Cross section of cauliflower leaf, showing intumescences formed after exposure to vapor liberated from one-half gm. of secondary methylamin chloride by adding 10 c. c. of a 15 per cent solution of sodium hydrate. A free-hand unstained section of one of the intumescences shown on Plate 62. Time of exposure to the vapor, 30 minutes. Temperature, 18°C. Date of collection, nine days after exposure. The section shows local thickening of the tissues with destruction of the chlorophyll. The dark parts of the section were green. ×75.

1
PITH CAVITY
2
3

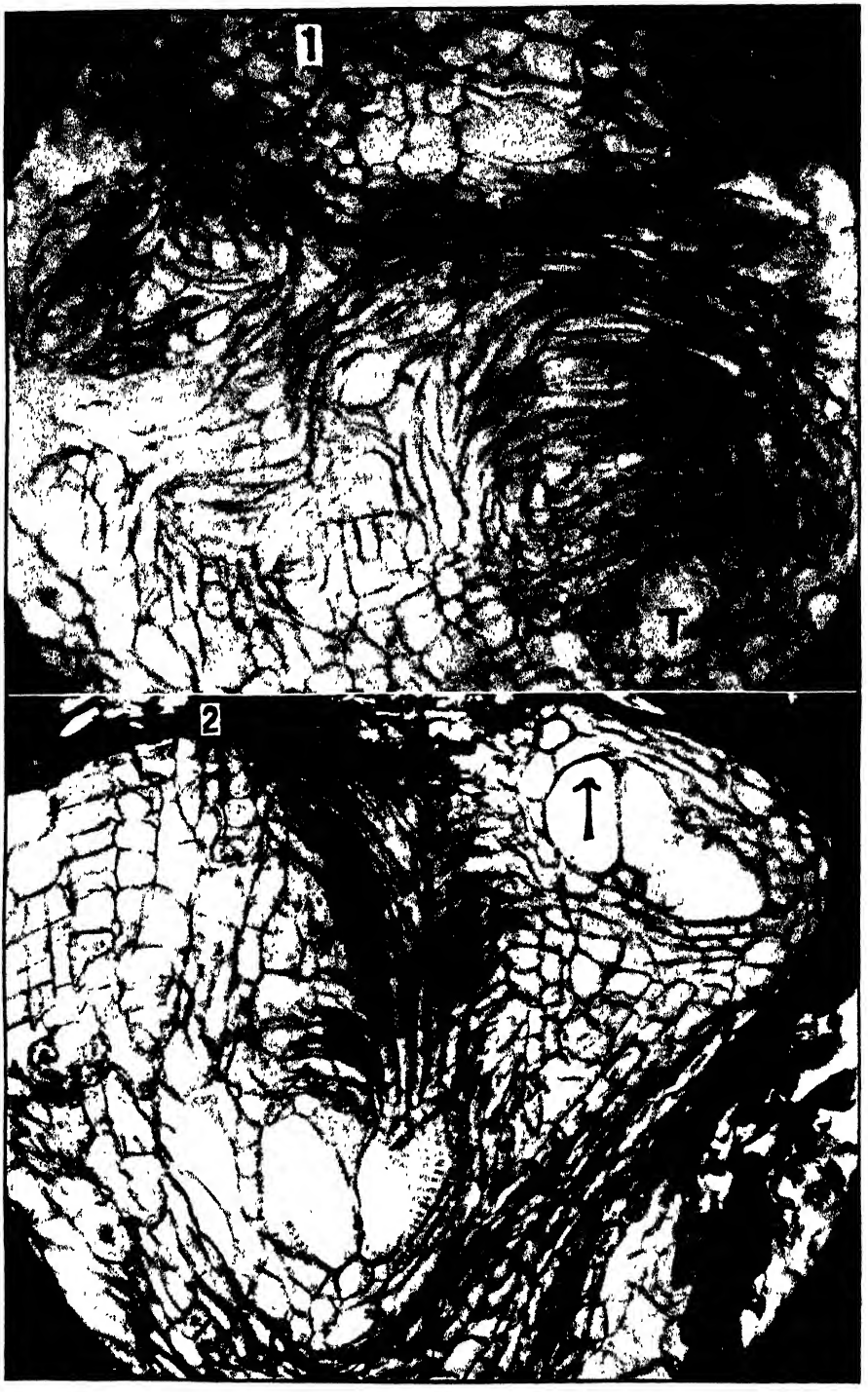

1
2

PLATE 47

1.—Vertical section of outer part of the proliferations just below the surface shown on Plate 5. Here are well-developed tracheæ (at *T*) and long, twisted cells (incipient vessels) strikingly suggestive of crowngall irregularities. ×160.

2.—Vertical section in same region as figure 1, but at another level, showing distorted vascular bundles in the new growth. The arrow shows the direction of the pith cavity, and the tissue at the top shows the beginning of the outermost proliferations—that is, those into the pith cavity. ×160.

PLATE 48

Portion of under surface of a cauliflower leaf showing intumescences due to vapor of ammonia. Time, 96 hours. The plant was exposed for 15 minutes at 20° C. to the vapor from 0.5 c. c. of aqua ammonia (sp. gr. 0.90) in 10½ cubic feet of air space, after which the plants were returned to the hothouse. The exposure was made at 11 a. m., and where no eruptions have appeared it may be assumed that the stomata were closed, particularly as the leaf was young. All of the outgrowths are on the lower face of the leaf, which is more abundantly provided with stomata than the upper surface, although there are also a great many on the latter. All of the smaller intumescences are still covered by a stoma-bearing epidermis. Exposed on August 28, 1916. Photographed on September 1. ×5.

1
2

PLATE 49

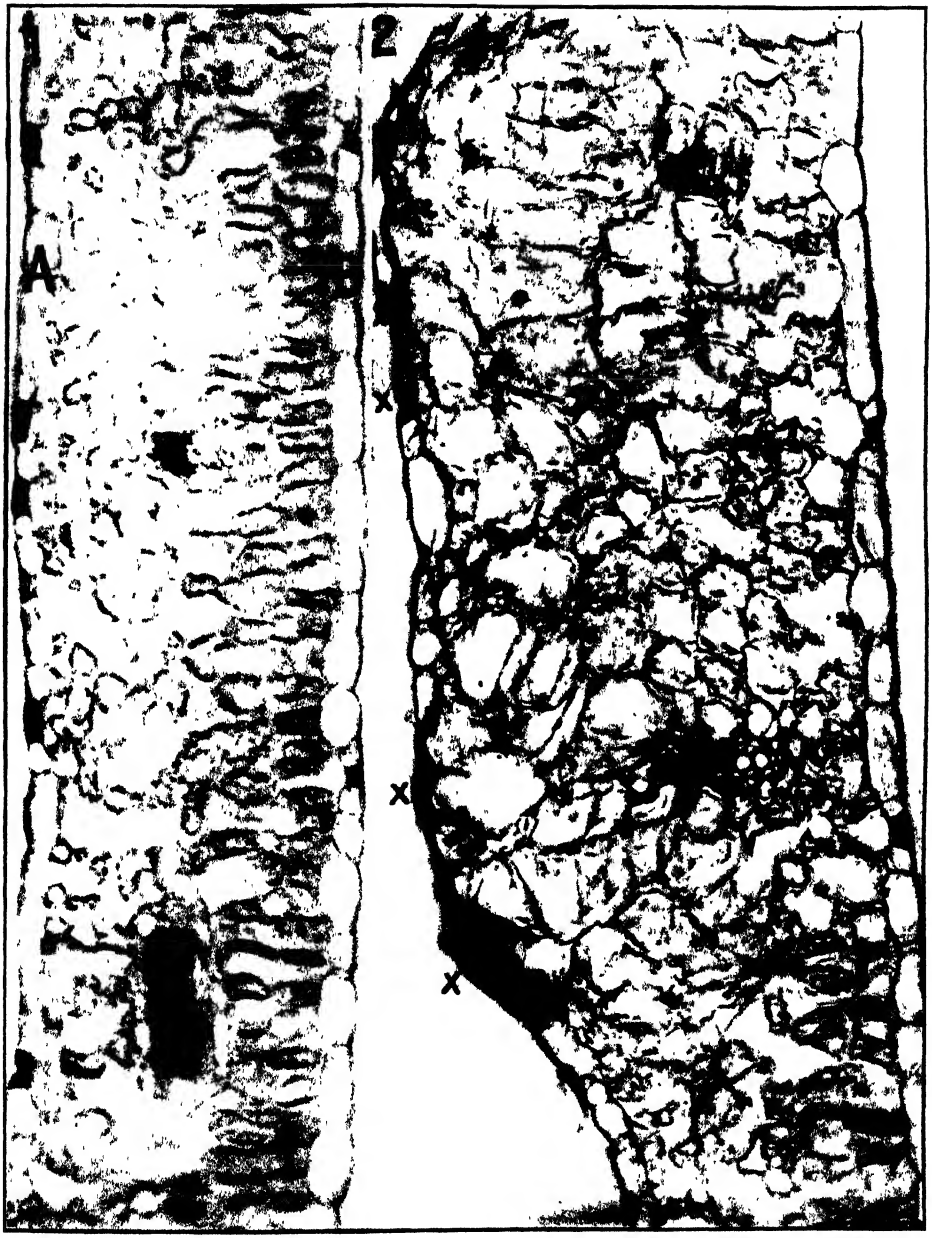
1.—Under surface of a cauliflower leaf from the same series as Plate 48 but nine days after 15 minutes' exposure to vapor of ammonia, and not from the same plant. Galls much larger and epidermis now ruptured in many of them. Most of the intumescences are on the under surface, and they occur in clusters. Probably the stomata on the upper surface of leaves, being exposed to the sun, close earlier in the day than those on the under surface, and those on the under surface undoubtedly close irregularly according to the needs of the various areas of the leaf. (For further evidence of this see Pl. 55.) The leaves became mottled light and dark green soon after exposure, but the phenomenon was fugitive—that is, two hours later they appeared to be normal. Photographed on September 6, 1916. × 5.

2 —Same series as figure 1, but at the end of 18 days, and from the upper surface of a leaf. Photographed on September 1, 1916. ×3.

PLATE 50

1.—Cross section of a normal cauliflower leaf. *A*, Lower surface; *B*, upper surface. Seven stomata are visible. In the center are two small vascular bundles, one cut longitudinally, the other crosswise. Section stained with methyl green and acid fuchsin. × 210.

2.—Early stage (third day) of cauliflower intumescence due to vapor of ammonia. The lower epidermis still covers the growing tumor and five stomata are visible in it (at *XX*). Same magnification and same leaf as in figure 1. Tumor formed out of the loose parenchyma. Particular attention is called to the increased size of the cells and the reduction of intercellular spaces in the intumescence, also to changes in the palisade tissue.


1
2
A
X
X
X

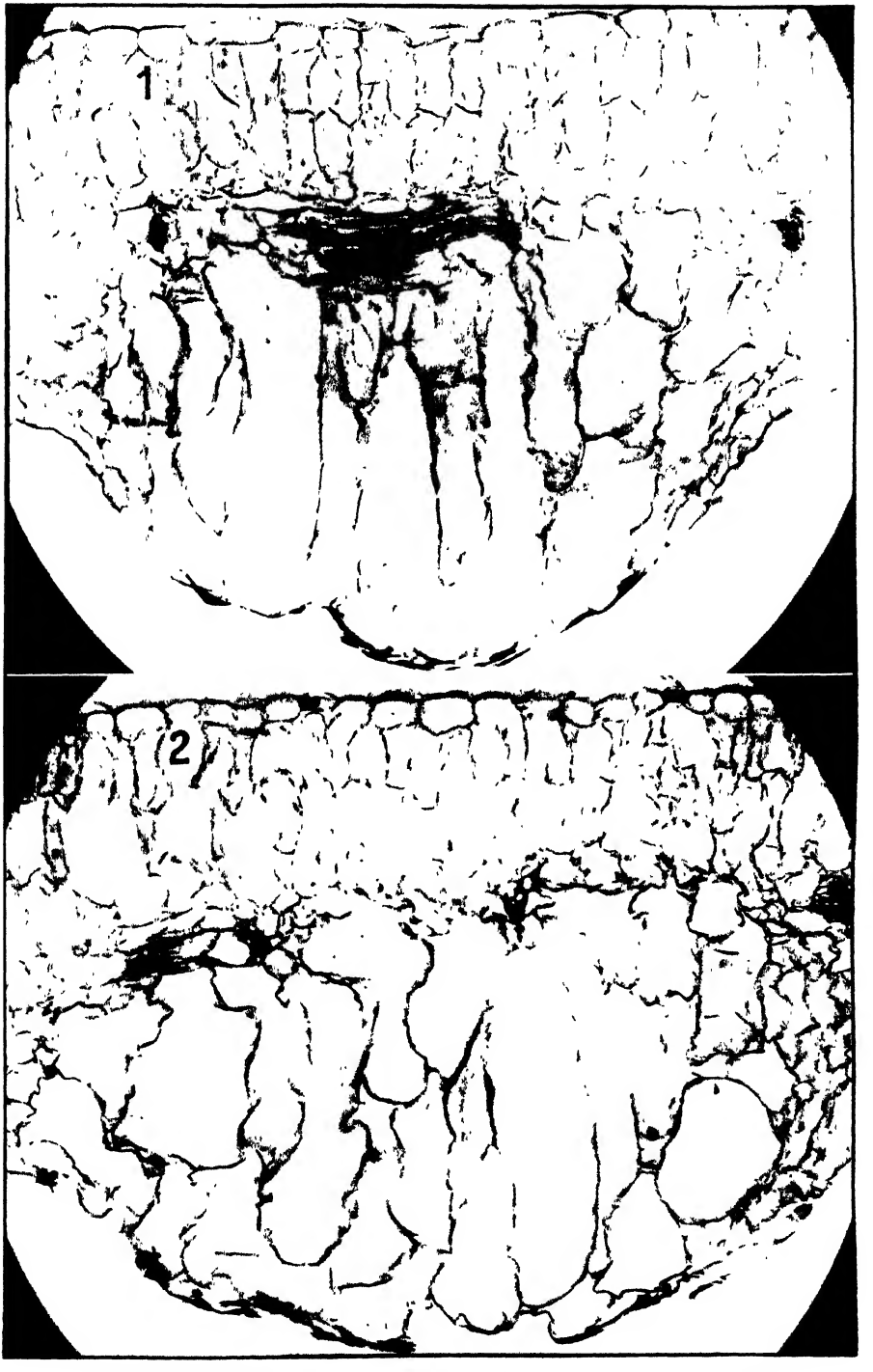
1
VB
2

PLATE 51

1, 2.—Free-hand unstained sections (water mounts) from old cauliflower intumescences caused by vapor of ammonia. The proliferation, which is much more compact than the normal tissue, is entirely or almost entirely from the loose parenchyma below the palisade tissue. The latter contains chloroplasts, but the intumescence does not. The vascular system (fig. 1 *VB*) appears to have been very little disturbed. Same series as Plate 50, figure 2, but much older. Exposed on August 28, 1916, sectioned and photographed on September 30. Most of the intumescences had shriveled by the end of the eighth week—that is, the stimulus was exhausted. ×160.

PLATE 52

1, 2.—Vertical sections of two ammonia-vapor intumescences on a cauliflower leaf showing an enormous hypertrophy of cells and no hyperplasia. The sections are from stained material which was fixed nine days after exposure to the vapor. Intumescences developed wholly from the cells of the loose parenchyma—that is, palisade tissue and vessels not involved. The volume of the largest cells in the tumor is a hundred times that of the normal cells out of which they have developed. These sections may be contrasted with those on Plates 51 and 57 to 61 which show hyperplasia. ×160.


1
2

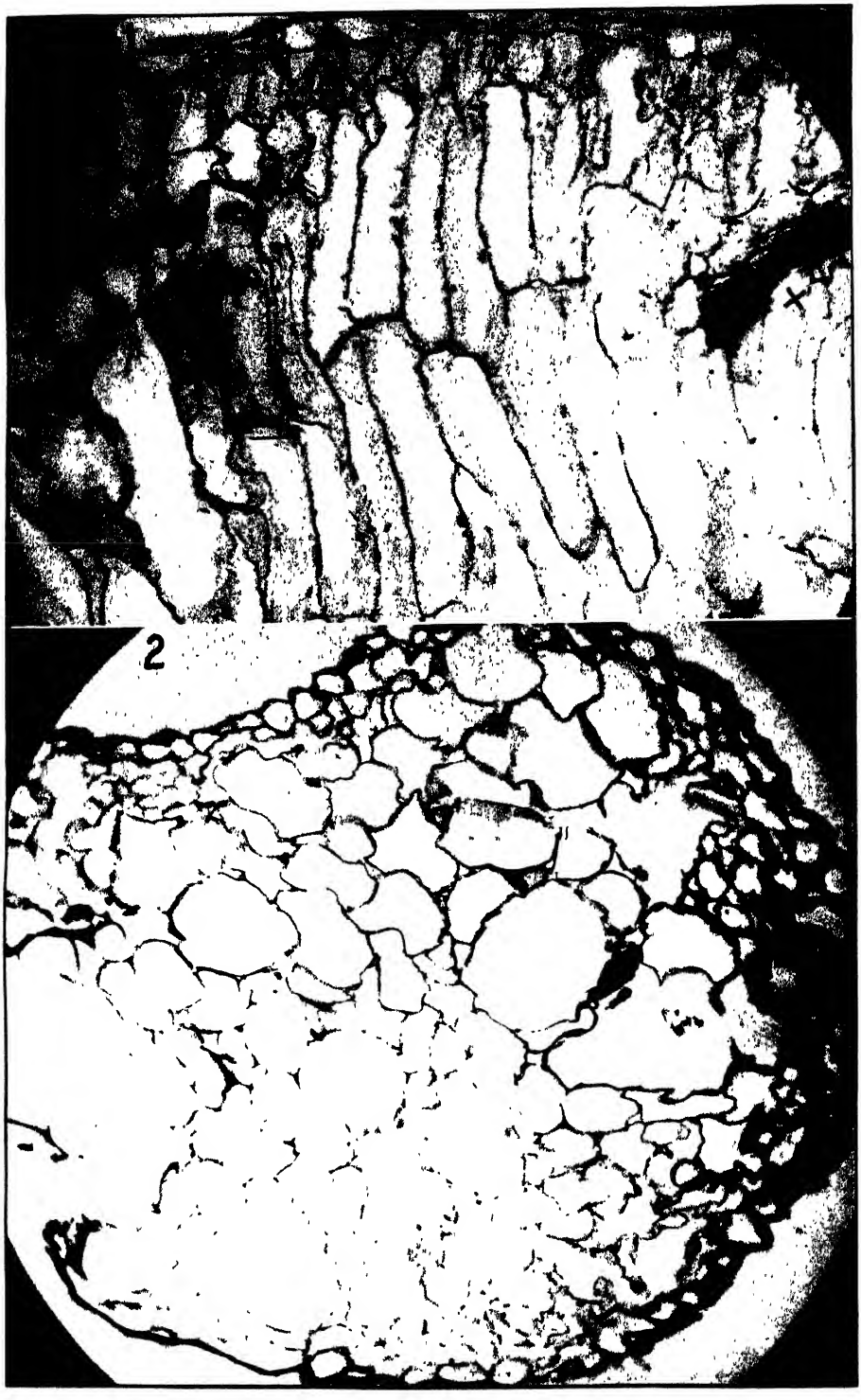

2

PLATE 53

1.—Like Plate 52 but here the palisade tissue is also involved. No evidence of hyperplasia nor much of ingrowths of vascular tissue (*X*). Two rows of cells at the lower surface of tumor omitted. Same magnification as Plate 52 (×160).

2.—Cross section of an ammonia intumescence above the level of the under surface of the leaf. Contrast with Plate 58, figures 1 to 4, which have the same magnification. Time, 9 days. × 160.

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PLATE 54

1, 2.—Cross sections through the deeper tissues of two ammonia intumescences in a cauliflower leaf showing that even in the depths they preserve their hypertrophic character. Time, nine days. The intumescences are surrounded by the normal tissues of the leaf. At *VB* are vascular bundles. Figure 1, × 160; figure 2, × 75.

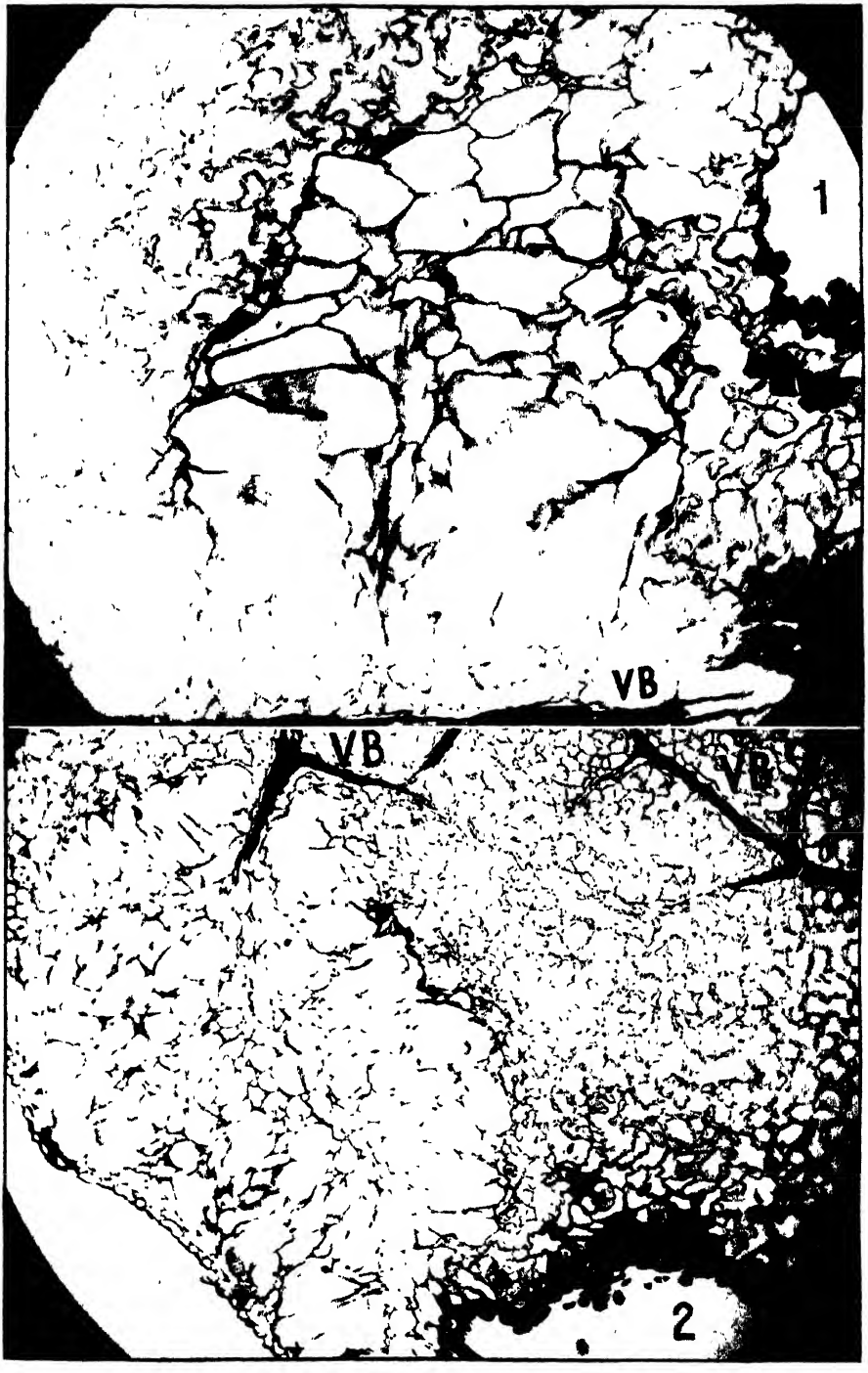

1
VB
VB
VB
2

PLATE 55

Cauliflower leaf showing killing effect of vapor of mixed ethyl alcohol and acetic acid (Carnoy's fixing fluid—three-fourths absolute alcohol, one-fourth glacial acetic acid) on maturer leaves of the same plant as Plate 56 The stomata were open presumably in the particular areas which have been killed (the white parts) and not in the other parts. The local killing effect was visible immediately. No intumescences appeared on such leaves, not even around the margins of the white spots. The other parts of the leaf remained normal in color and function. Exposed on September 21, 1916. Photographed on September 28. ×1½.

PLATE 56

Under surface of a cauliflower leaf showing intumescences produced by the vapor of mixed ethyl alcohol and acetic acid (crowngall products). Leaf young when exposed and the stomata closed or nearly closed, so that only a very little of the vapor could enter. The plants were inclosed in 10½ cubic feet of air space at 20° C. and exposed for 30 minutes on September 21, 1916, at 1 p. m., to the vapors from 10 c. c. of the fluid in a porcelain capsule floating in a dish of water at 65° C. Only a small amount of the fluid was vaporized but enough to be smelled. Older leaves (half grown) were killed quickly in many irregular spots (Pl. 55), but leaves of this age did not show any immediate injury. The height of the intumescences may be judged by their shadows. Occasionally several are fused. Some are too small to show on the plate. None developed on the midrib or on the main veins and none or very few on the upper surface. Other leaves, where a greater number of stomata may be assumed to have been slightly open, showed hundreds of tiny coalescent intumescences, the under surface in large patches being one mass of eruptions. Photographed on September 28. ×5.

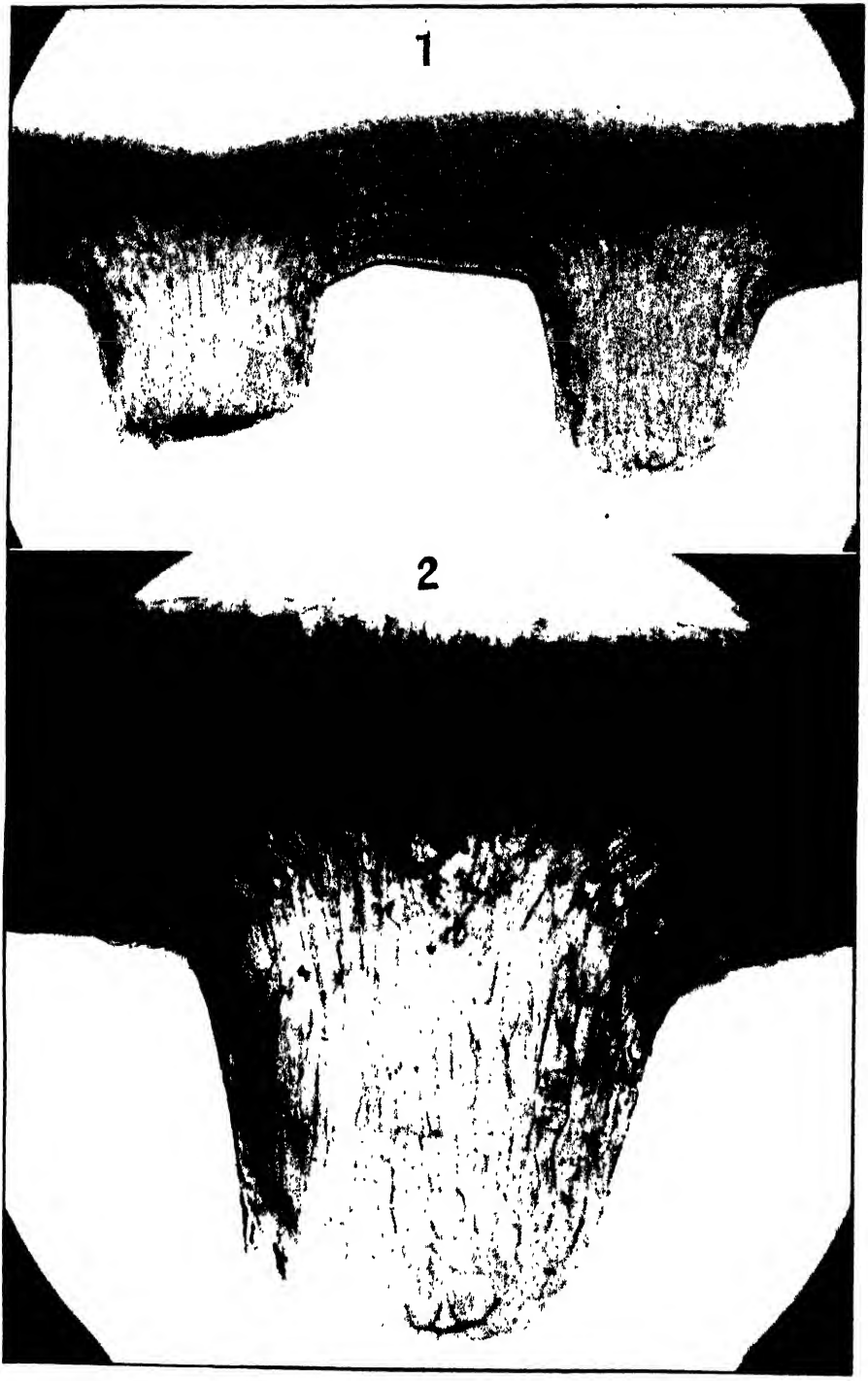

1
2

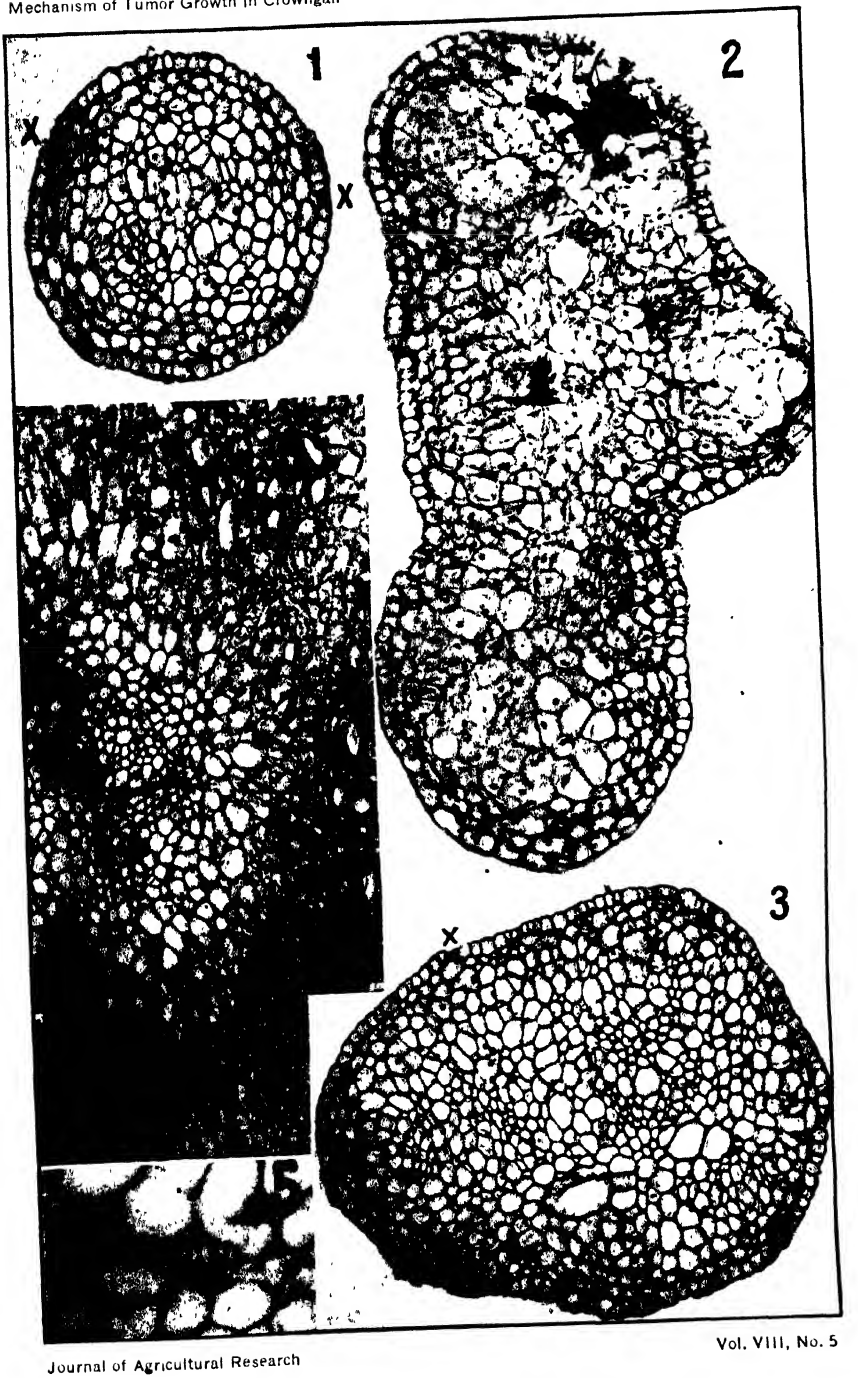
PLATE 57

1.—Cauliflower intumescences due to vapor of mixed ethyl alcohol and acetic acid (Carnoy's fixative) Free-hand unstained section (water mount) from the leaf shown on Plate 56. The growths are unlike many of those due to vapor of ammonia—that is, they are all hyperplasias. The tumor tissue is much more compact than the loose parenchyma from which it has originated, is free from chlorophyll, and is not endowed with continuous growth. × 75.

2.—This is an enlargement of the right-hand tumor shown in figure 1. The darker parts, at least in the normal portion, represent chlorophyll-bearing tissue. Exposed on September 21, 1916. Photographed on September 28. × 160.

PLATE 58

1 to 5.—Cross sections from fixed and stained portions of the little tumors which developed on the leaf shown in Plate 56. Each one is bounded by an epidermis, and in places stomata are visible, as at *X*. The smaller cells in each, best seen in figure 3, are cross sections of incipient vascular bundles. Figure 4 is a section just below (inside) the lower surface of the leaf. At the bottom is epidermis showing stomata. On the right is unchanged loose tissue of the leaf out of which the hyperplasia has developed. This shows large intercellular spaces. In the middle at the left is the compact tissue of the tumor, while above it is transition tissue. Observe absence of intercellular spaces in the tumor and the beginnings of spiral vessels. × 160. Figure 5 shows spiral vessels enlarged from the center of the tumor in figure 4. ×640.


1
X
X
2
3
X

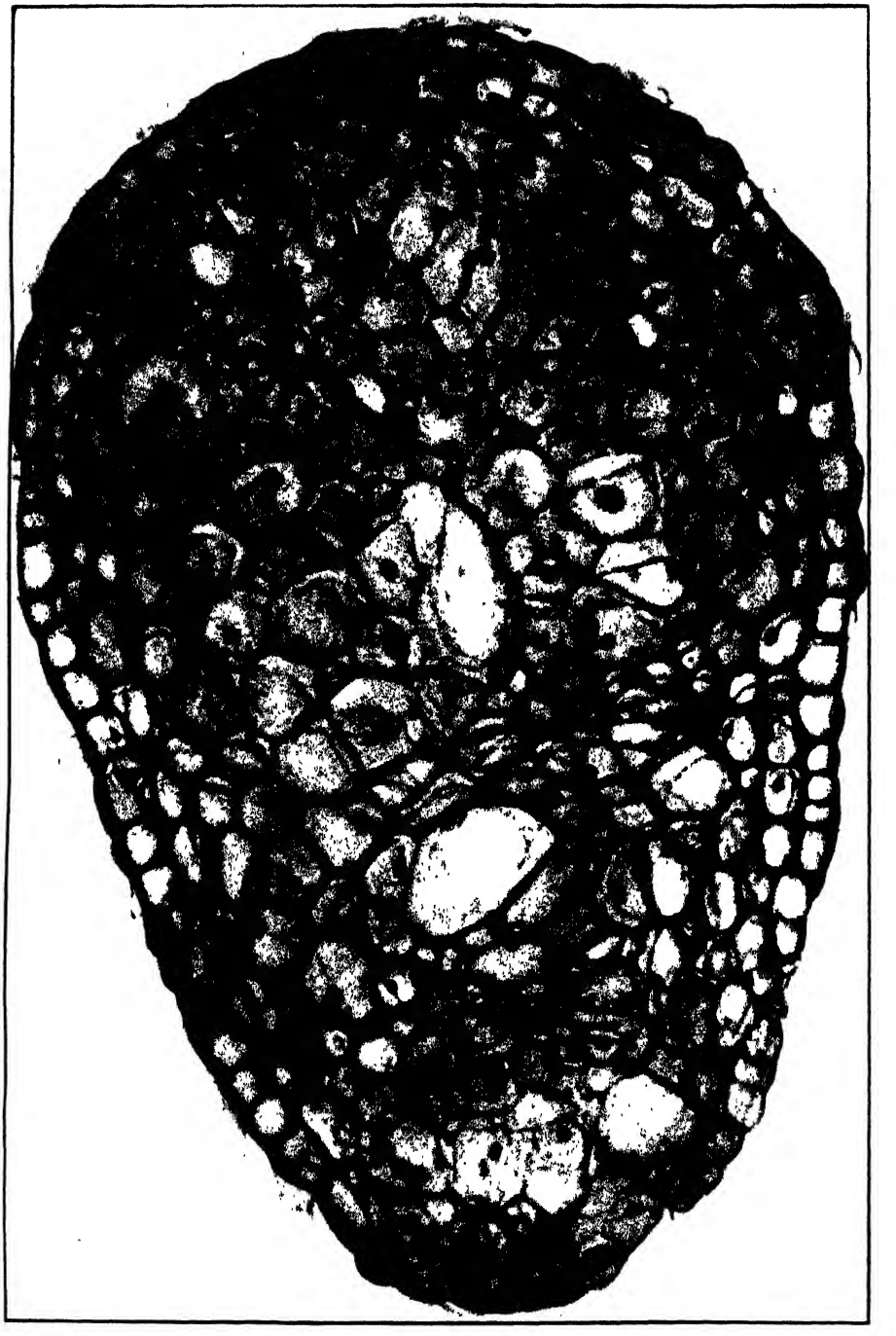


PLATE 59

Cross section of one of the intumescences of Plate 56 further enlarged to show mixed hypertrophy and hyperplasia as in crowngall. The round, dark spots in the cells are nuclei. This had the same initial magnification as Plate 58, figures 1 to 4, but the photograph was enlarged three times by the engraver. ×480 (circa).

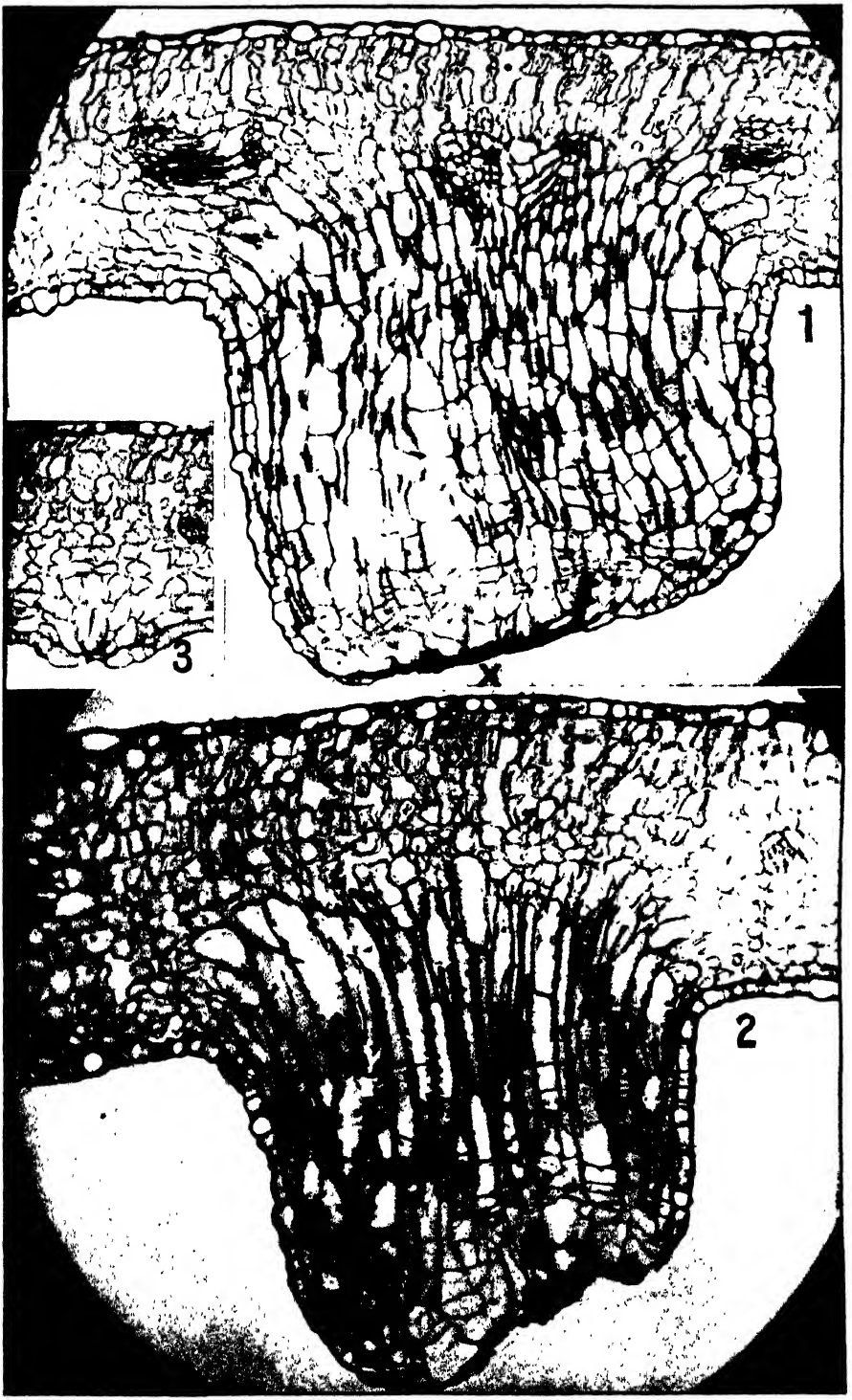
PLATE 60

Vertical sections from fixed and stained portions of cauliflower intumescences shown on Plate 56 and in cross section on Plates 58 and 59. Time, 7 days. Upper surface of leaf at top.

1.—Middle vascular bundles giving off incipient vessels to the tumor. The epidermis is dead at *X*, and a cork layer has formed under it. × 160.

2.—Sieve tubes and incipient xylem vessels developing. Here again the epidermis covering the top of the hyperplasia is dead, and cork has formed. × 160.

3.—Section through the middle of an intumescence which received a *minimum* of stimulation and in which only the epidermis and two layers of cells immediately under it have responded. Time, 7 days. Many such abortive tumors can be seen on the surface of the photograph from which Plate 56 was made and some of them also in the half-tone. ×160.


1
3
X
2

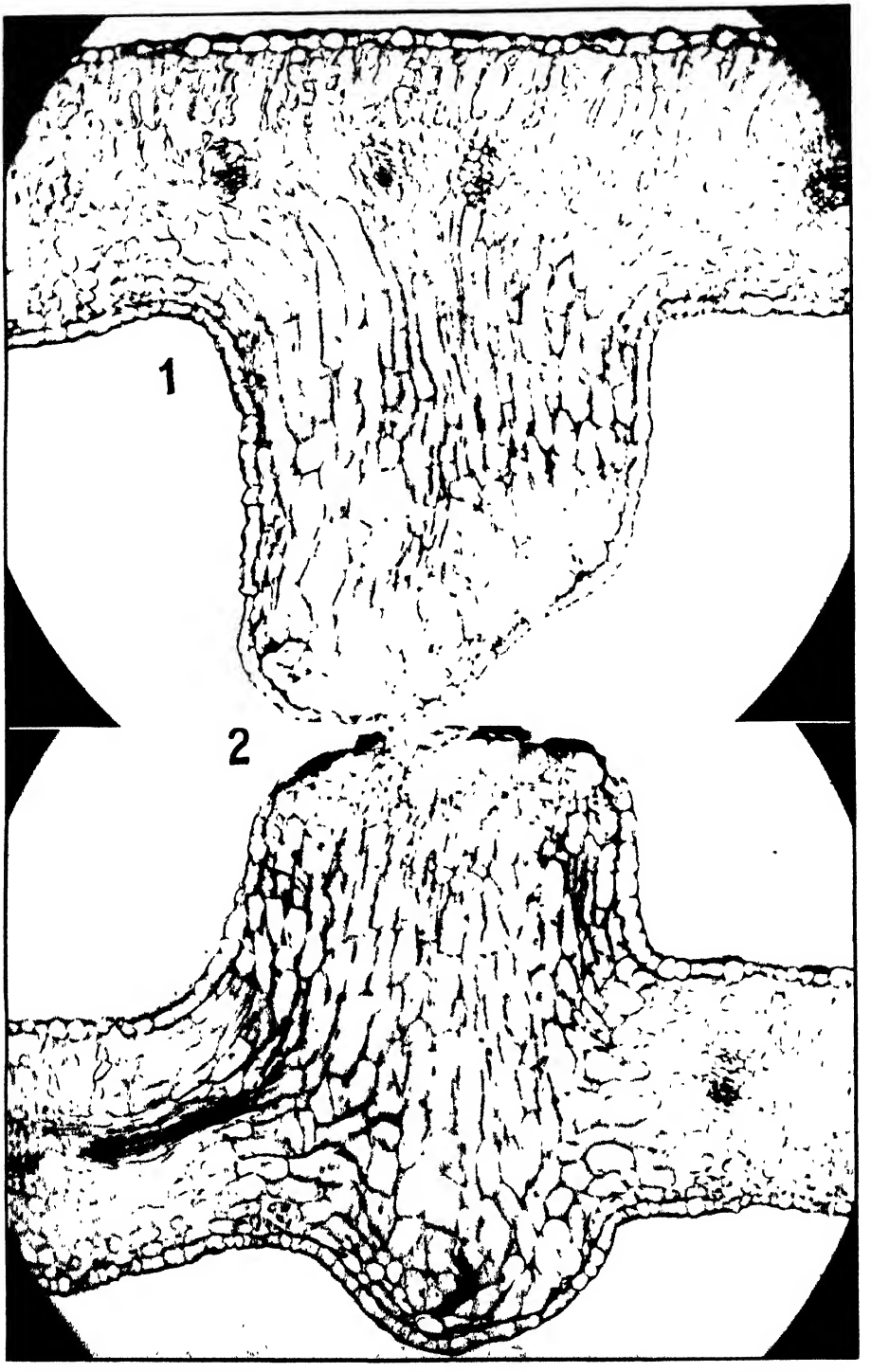

1
2

PLATE 61

1.—Like figures 1 and 2 of Plate 60, but with the intumescence still covered by an epidermis and free from cork. Incipient vessels can be seen entering the tumor from the leaf bundles. Upper surface of leaf at the top. × 160.

2.—Alcohol-acetic-acid intumescence in which entire thickness of the cauliflower leaf is involved. In this instance the larger part of the growth is from the palisade tissue (at the top). × 160.

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PLATE 62

1, 2.—Intumescences formed on a cauliflower leaf exposed 30 minutes in 10 1/2 cubic feet of air space to vapor liberated from one-half gm. of secondary methylamin chlorid by adding 10 c. c. of a 15 per cent solution of sodium hydrate. Temperature, 18° C. Time, 2 p. m. Collected nine days after exposure.

1.—Under surface of leaf corresponding to figure 2.

2.—Upper surface of same leaf. Many of these intumescences involved the whole thickness of the leaf; others appeared only on the under side, or only on the upper side. In figure 1 the unruptured swellings at *X*, *X*, correspond to ruptured tumors on the other surface of the leaf. × 3 1/2 nearly.

Inasmuch as it is probably more or less difficult for the chemist to obtain an amin chlorid entirely free from ammonium chlorid it is possible that we have here to deal with mixed stimuli. This can not be stated positively until further experiments are made. My reason for thinking so is that in addition to the fishy odor of the dimethylamin, the odor of ammonia was also detected, especially when the nose was brought close to the capsule containing the mixture.

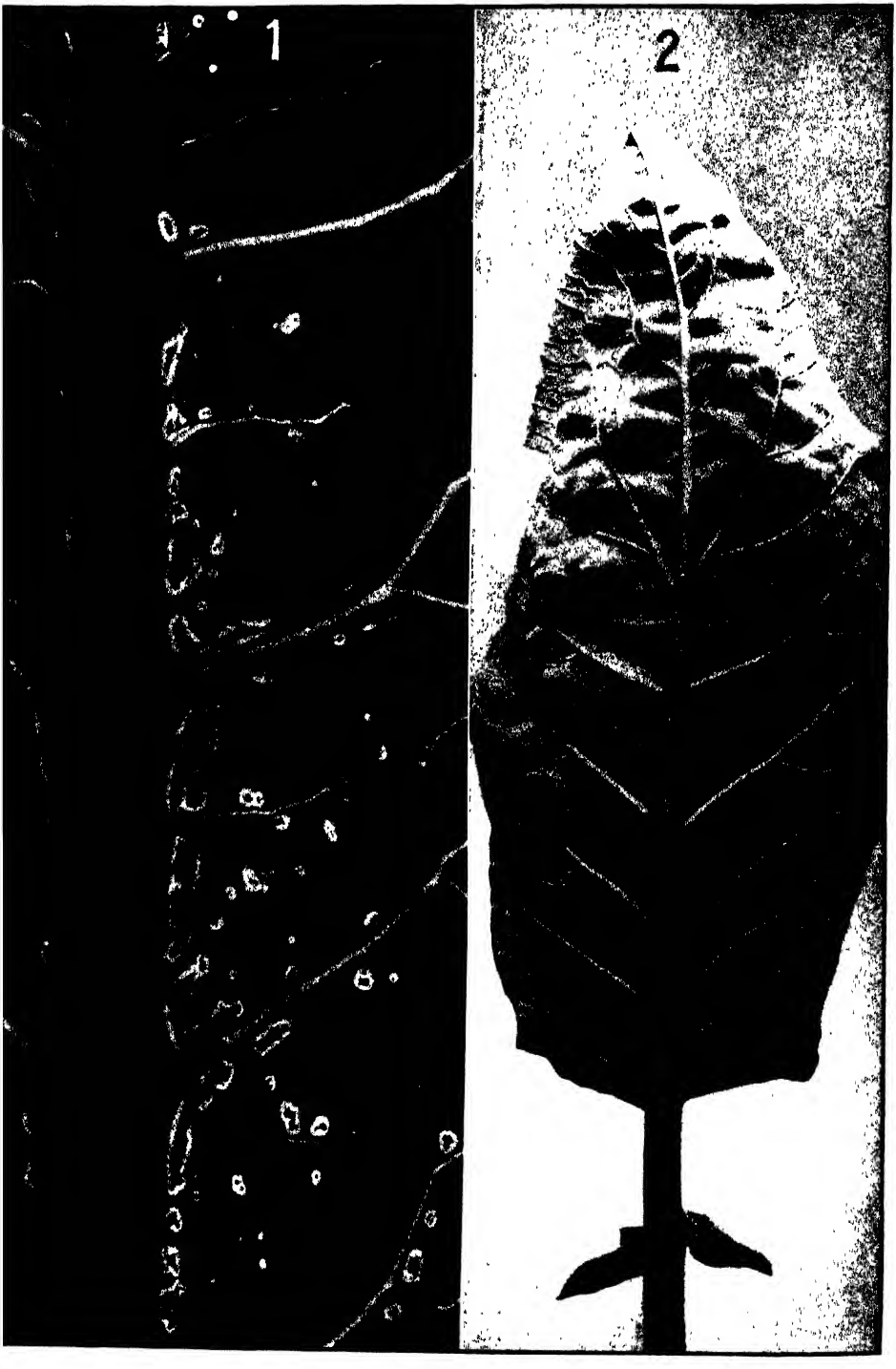

1
2

PLATE 63

1.—Same as Plate 62 but photographed by transmitted light to show the opaque centers of the intumescences due to death of tissues and the chlorophyll-free translucent borders which allowed the light to shine through readily, and therefore are white in the photograph. All the larger intumescences are ruptured. ×3 1/2 nearly.

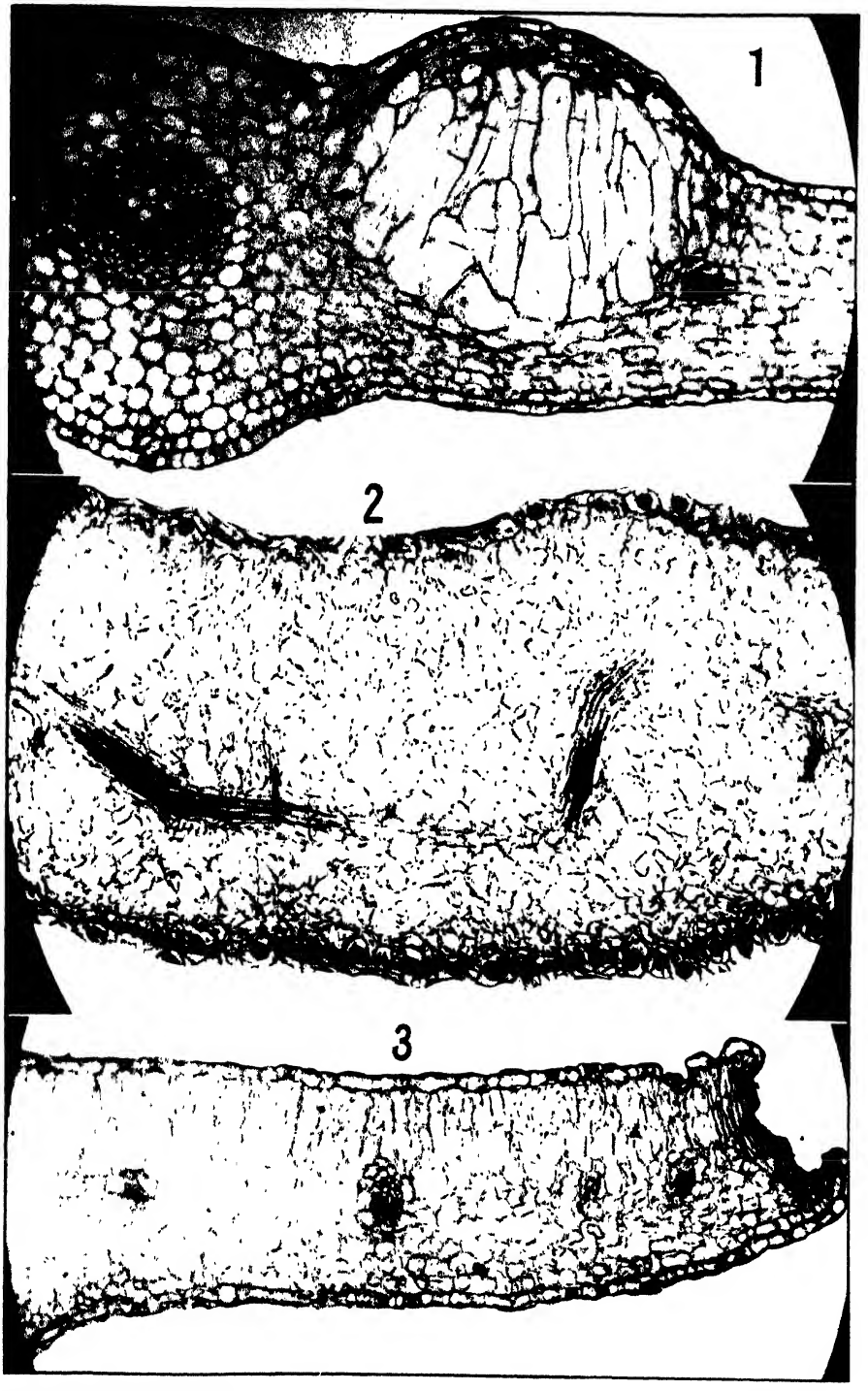
2.—Under surface of one of many leaves of a cauliflower plant exposed to vapor of primary ethylamin on December 5, and photographed December 14, slightly less than natural size. These swellings and bucklings of the parenchymatic tissue were not present when it was exposed and are possibly to be attributed to the exposure. The experiment was made by liberating the vapor from one-half gm. of primary ethylamin chlorid in 10 1/2 cubic feet of air space by adding 10 c. c. of a 15 per cent solution of sodium hydroxid. Eighteen leaves on the exposed plant showed the phenomenon, the domes of the curvatures being directed toward the lower surface of the leaf. These parts are thicker than the normal parts of the leaf and their palisade tissue has given place to round-celled parenchyma (Pl. 64, fig. 2). In a repetition of the experiment 5 leaves showed the disease on one plant and 12 on another.

PLATE 64

1.—Paraffin-embedded stained section of cauliflower leaf exposed to secondary methylamin showing hypertrophy of interior cells, absence of chlorophyll, and enlargement of the nuclei. At the left, cross section of a normal leaf rib, and at the right a longitudinal section of a smaller group of vascular bundles. Same material as Plate 46, figure 3. ×160.

2.—Cross section of cauliflower leaf (Pl. 63, fig. 2) exposed to primary ethylamin showing general thickening to double the normal thickness and absence of palisade tissue (upper surface of leaf at top.) ×160.

3.—Same leaf and section as figure 2, but from a normal portion. Palisade tissue above. ×160.


1
2
3

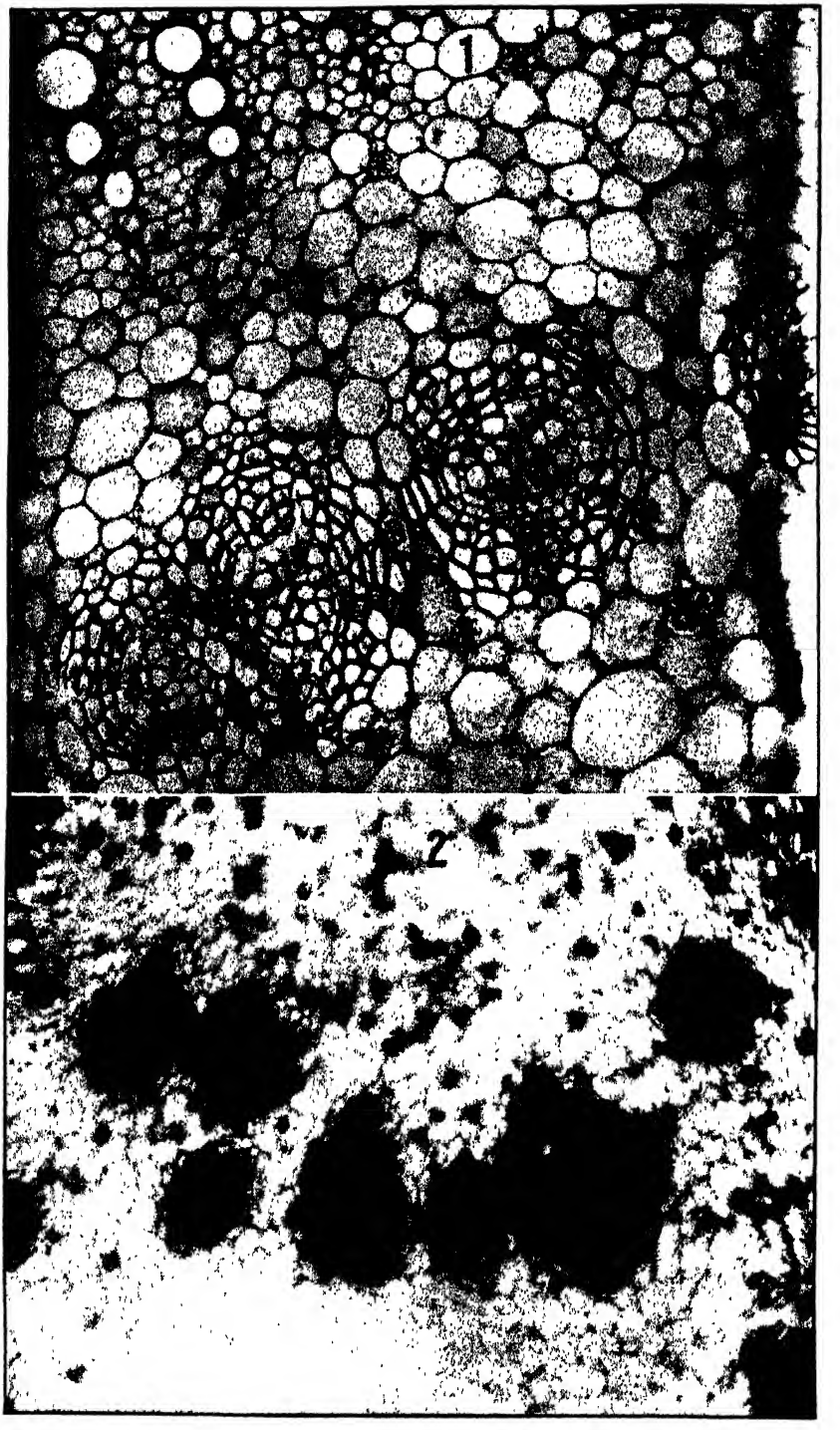

1
2

PLATE 65

1.—Portion of ring of concentric medullary bundles in the axis of inflorescence of *Ricinus communis*. Ordinary vascular tissue at the top. Safranin stain. ×160.

2.—Group of concentric medullary bundles in a branch-gap of *Ricinus communis*. Ordinary bundles (out of focus) at top (right and left) on edges of the gap. Section is free-hand and unstained. ×75.

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SOROSPORELLA UVELLA AND ITS OCCURRENCE IN CUTWORMS IN AMERICA

[PRELIMINARY PAPER]

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In 1888 Sorokin (5)[1] described and illustrated as a parasite of the cutworm *Agrotis segetum* Schiff., in Russia, a fungus which he called *Sorosporella agrotidis*, employing thereby a new generic as well as a new specific

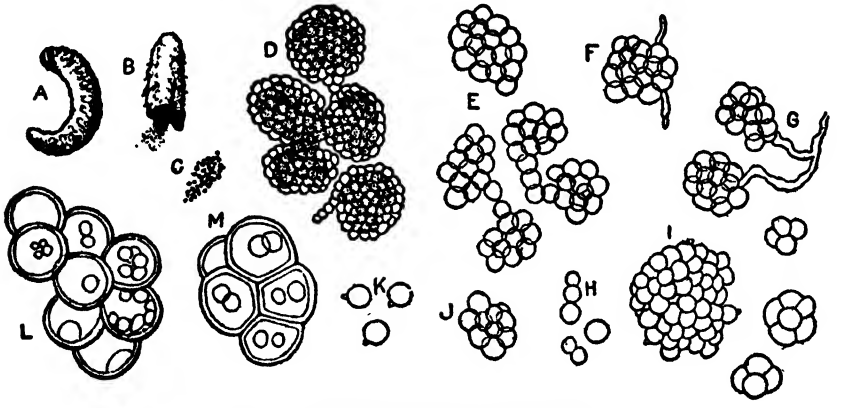

FIG. 1.—*Sorosporella agrotidis* (after Sorokin).

name. The descriptive generic name was chosen because of the presence in infected insects of grapelike masses of spores, masses which reminded Sorokin of similar formations in the genus Sorosporium of the Ustilaginales, although he suggested no relationship between the two forms. For purposes of comparison, a copy of Sorokin's illustration is presented (fig. 1) which, together with his diagnosis, is sufficiently complete to render an identification of the organism possible.

Sorokin had but few diseased larvæ, and did not, therefore, attempt inoculation experiments; nor did he study at any length the life history

[1] Reference is made by number to "Literature cited," p. 194.

of the organism. As a consequence, subsequent writers have been at a loss as to the systematic position of the genus Sorosporella. Several have gone so far as to suggest its affinity to the Entomophthorales, basing their claims on its apparent resemblance to the genus Massospora Peck, which Forbes and Thaxter have shown to be entomophthoraceous.

In 1889 Giard (2) translated Sorokin's article into French, and in the same issue of the publication followed it by a note in which he pointed out that *Sorosporella agrotidis* Sorokin was undoubtedly identical with *Tarichium uvella* Krassilstschik. Giard reached this conclusion because of the many points of similarity between the two forms, such, for example, as the reddish color of the fungus mass when observed with the naked eye, the peculiar internal development of both forms, the comparative uniformity of the spores in form and size (Sorokin, 4 to 7μ; Krassilstschik, 8 to 10μ), and the existence of papillæ on the spores. He furthermore called attention to the fact that the specific name "*uvella*" of Krassilstschik served also to indicate this peculiar massing of spores, a condition entirely dissimilar to that found in other entomogenous forms.

The generic name Tarichium, if valid at all, is applicable to those forms of Entomophthorales in which resting spores only are known. As will be shown later, the fungus under consideration is in no way connected with this group, and, therefore, the use of the name Tarichium as employed by Krassilstschik (4) becomes invalid. Hence, the correct name for the organism is *Sorosporella uvella* (Krass.) Gd., Giard having first recognized the form described by Sorokin as identical with that described by Krassilstschik.

Early in June two larvæ and one pupa of a cutworm (*Euxoa tessellata* Harris) which had died in breeding jars were given to the writer. The insects originally came from College Park, Md. No fungus was visible externally, but upon breaking open the larvæ (Pl. 66, *A*) a reddish brown powdery spore mass was seen which completely filled the interior of the insect's body. Microscopic examination demonstrated that the fungus was none other than *Sorosporella uvella* (Krass.) Gd.

So far as the writer is aware, there are no records of the appearance of this fungus in Europe since 1888; and, furthermore, this seems to be the first published account of its occurrence in America, except for a brief note of its presence in Ottawa, Canada, by Gibson (1).

The object of the present preliminary note is merely to record the presence of this entomogenous form in the United States and to present some evidence to show that it is not entomophthoraceous, deferring for a time a more complete account of its life history as well as a consideration of its efficacy as a fungus parasite of insects.

In the discussion which follows, the large, spherical, thick-walled cells will be spoken of as spores and the aggregations of these cells as spore masses. These terms have been used advisedly. It is realized that so

long as the method of formation of these bodies is unknown such usage is not entirely justified. At the present time, however, these names are perhaps as convenient and acceptable as any.

As noted above, the vegetative development of the organism is apparently completed within the body of the insect, the growth almost wholly replacing the body contents. Under normal conditions, the chitinous body wall of the insect remains intact, becoming dry and brittle, translucent, and very much wrinkled. The body does not, however, become mummified as do the bodies of larvæ infected with species of Cordyceps. On the contrary it is loosely filled with quantities of spores and resembles a minute sac filled with dust.

The fungus consists of large irregular masses (Pl. 66, *B*, *C*), which in turn are composed of numerous spherical sporelike elements that are firmly attached to one another. These spore aggregations vary greatly in size and form, but the elements of which they are composed are quite uniform in size and structure. Upon examining a large number of crushed spore masses, however, it would appear at times that the peripheral cells were, on the whole, somewhat larger than the inner ones, but at the present time it is not possible to say that this difference invariably exists. A further difference exists in the germinative power of the outer and inner cells. This condition may be readily seen if a crushed spore mass is mounted in a hanging drop of water. Those cells or pseudospores which were originally internal remain inactive, while those spores which were external produce germ tubes in due time. In other respects the individual elements are similar. They are nearly spherical in form, have rather thick walls, are colorless when viewed individually, and measure from 7 to 10μ in diameter.

As already stated, the spores can be separated from one another only with great difficulty, and on crushing a spore mass ruptured spore walls are often seen attached to uninjured spores (Pl. 66, *E*, *a*).

Sorokin and Krassilstschik both speak of the presence of papillæ on the spores, and Sorokin also observed a mycelium in connection with the spore masses (fig. 1). The same author suggested that the papillæ were peduncles to which the mycelial filaments had been attached, yet thus far no fungus mycelium has been seen within the insect's body, although papillæ have occasionally been observed (Pl. 66, *E*, *b*). At the present time, therefore, the manner of formation of the spore masses is unknown, but as no mycelial filaments have been observed, and as the above-mentioned papillæ have been seen, though rarely, it seems probable that the spore masses may arise through the successive budding of the original elements. It will be necessary, however, to examine cutworm larvæ in early stages of the disease or to obtain some evidence by artificial culture before this point can be proved.

Up to this point, the writer's observations confirm those of Sorokin, who was, however, unable to pursue the study further, owing to lack of

material. Krassilstschik, according to Thaxter (7, p. 190) in his description of *Tarichium uvella*, states that in nutrient fluid the large spherical spores germinate and give rise to septate hyphæ that ultimately produce single, terminal, cylindrical colorless spores, measuring 9 by 3μ. Although Krassilstschik's description is meager and unaccompanied by figures, it would seem that this process agrees essentially with that described below.

Several more infected insects were received in July, so that enough material was available to conduct germination and cultural tests. At the end of 48 hours in hanging-drop water cultures each of the peripheral cells of the spore masses gives rise to one germ tube. These are at first simple, sometimes septate, and on the third or fourth day each produces a single cylindrical spore at its tip (Pl. 66, *G*). These spores, which may be called secondary spores, are entirely unlike those from which they arose, being colorless, thin walled, and cylindrical. Their measurements vary from 4 to 6μ in width and 9 to 11μ in length. Two large vacuoles are invariably present, one at each end of the spore. The germ tubes gradually lengthen somewhat and branch sparingly. The ultimate branches are often terminated by bottle-shaped portions that show, or at least have a tendency toward, verticillate grouping. At the end of 10 days growth ceases, owing apparently to the exhaustion of the nourishment supplied by the large spherical spores. During the latter part of this period the secondary spores are cut off successively from the tips of the sporophores, and in hanging-drop water cultures adhere to one another after abjunction (Pl. 66, *H*).

The promycelium-like sporiferous filaments arising from the large spherical cells are irregularly septate, limited in growth, and measure from 3 to 5μ in diameter.

From the above description it will be seen that the fungus under consideration has no characters in common with the Entomophthorales. The cœnocytic elements of growth of the latter, together with the method of spore formation and discharge, are in no way comparable to analogous processes in Sorosporella.

It is not at all certain whether the inner cells of the spore masses function as reservoirs of food for the outer germinating spores, as is supposed to be the case in some bulbils, because, judging from their staining properties after several days in hanging drops of water, there is apparently no reduction in the amount of their cell contents. Furthermore, while the individual elements are firmly fixed to one another, a connecting passage between them, although perhaps not necessary for the transfer of protoplasmic material, has yet to be demonstrated.

Attempts to cultivate the organism have not thus far yielded entirely satisfactory results, probably owing to an improper choice of nutrients. In the present preliminary trials only potato agar and oat agar were

employed, in both of which growth is exasperatingly slow. The outer cells of the spore masses, however, when plated in such media, give rise to a mycelium a portion of which is represented on Plate 66, *N*. No spores have as yet developed in such cultures, although they are now several weeks old.

No attempts have been made to study the development of the secondary spores or conidia in culture media, but their germination in water has been observed (Pl. 66, *M*, *O*).

From the studies thus far conducted it seems probable that the large, spherical, thick-walled cells function as resting spores, which in this fungus are closely attached to one another in masses. Such a condition is, as noted by Sorokin, very similar to that found in certain smuts, such as Urocystis and Tubercinia.

The early stages of germination of the spore balls of such smuts and of the spore masses of Sorosporella are also similar. The later development of the germinating spore masses of Sorosporella, however, more closely resembles that of the verticillate Hyphomycetes (Pl. 66, *H*, *K*, *L*). The branched sporophores ending in bottle-shaped tips and the abjunction of the typical conidia which adhere to one another in groups as they are cut off suggest this relationship.

While the spore masses, as mentioned above, bear a certain superficial resemblance to the spore balls of Ustilaginales, they nevertheless more closely resemble that class of propagative bodies known as bulbils, which are known to occur in the life histories of certain Ascomycetes and Basidiomycetes.

Hotson (3) and others have shown that bulbils are often directly associated with hyphomycetous growths. In some cases, as in *Papulospora aspergilliformis* as figured by Hotson, the bulbils upon germination give rise to typical hyphomycetous forms. In other cases the same mycelium produces bulbils and conidia at different places.

What the early stages in the development of Sorosporella are in the insect is not known. The presence of papillæ on the spores suggests that vegetative development is carried on by a budding process, but, as mentioned above, this point has yet to be proved. It is known, however, that finally the entire larval body is filled with the above-described large spore aggregations, which will undoubtedly be incorporated in the soil when the larvæ disintegrate. In a moist chamber such spore aggregations give rise to the secondary spores mentioned above, and it may be assumed that the same process takes place in the soil. In such a position the secondary spores will readily be able to infect fresh cutworms, which habitually burrow in the earth.

Thus far no experiments have been conducted to test the parasitism of *Sorosporella uvella*, but it is hoped that these experiments, as well as a more complete study of the life history of the organism, will be accomplished soon.

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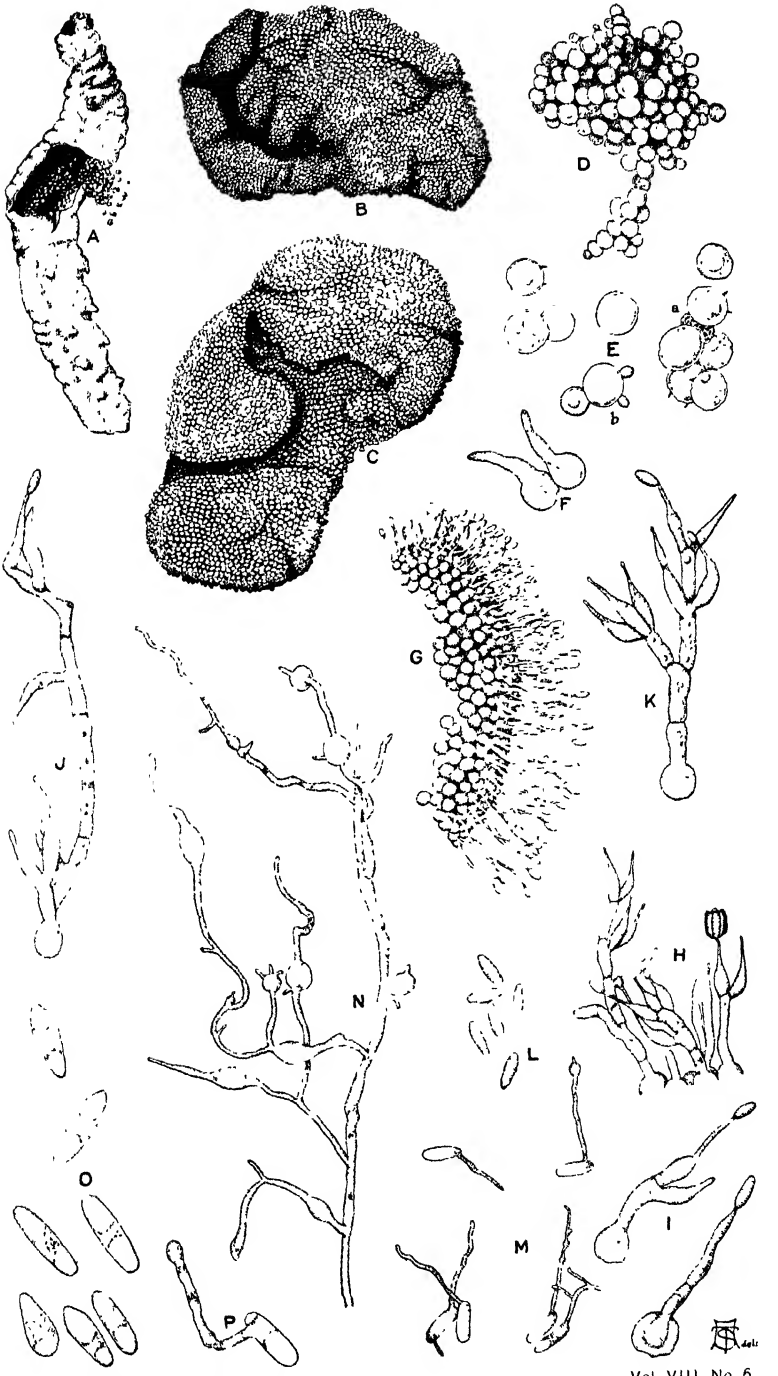
PLATE 66

Sorosporella uvella.

A.—Larva of *Euxoa tessellata* infected with *Sorosporella uvella*. ×1.2.
B, C.—Spore masses. ×120.
D.—A portion of a spore mass. ×252.8.
E.—*a*, Spores with ruptured walls of other spores attached. *b*, Spores showing papillæ. ×580.
F.—Germinating spores. ×580.
G.—A portion of a spore mass showing peripheral germination. ×252.8.
H, K.—Spores germinating, in part showing verticillate branching. ×580.
L.—Secondary spores. ×580.
M.—Secondary spores germinating. ×580.
N.—A portion of vegetative mycelium from a culture. ×580.
O.—Secondary spores. ×1080.
P.—Secondary spores germinating. ×1080.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MEASUREMENT OF THE INACTIVE, OR UNFREE, MOISTURE IN THE SOIL BY MEANS OF THE DILATOMETER METHOD

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INTRODUCTION

One of the problems in the domain of soil physics which still awaits a satisfactory and comprehensive solution is the relation that exists between the soil and the soil water. In this relation there are included two important and fundamental questions: (1) The effect of the soil upon the soil moisture and (2) the condition in which the moisture exists in the soil.

In the present paper there are presented two methods—the freezing-point and the dilatometer methods—which appear to give very valuable information concerning these two important and fundamental questions. The freezing-point method yields only qualitative results, while the dilatometer method gives quantitative data.

HISTORICAL REVIEW

In determining the lowering of the freezing point of soils (2) [1] by the freezing-point method at very low and very high moisture content it was found that in all soils, with the exception of quartz sand and some extreme types of sand, the lowering of the freezing point increased at a far greater rate than the percentage of water decreased. In other words, the ratio of the freeezing-point depression and the percentage of water content were not inversely proportional (approximately) as might be expected, save only in the quartz sand and some of the extreme types of sand. Thus, a clay at 92.76 per cent of moisture gave a depression of the freezing point of 0.039° C. and at 39.28 per cent 1.075° C. The ratio of the percentage of water at the two moisture contents is only 2.37, while that of the depression is 27.56.

This phenomenon was explained on the supposition that some of the water contained by the soils was either physically adsorbed or loosely chemically combined or both, in which event this portion of the water was not free or active to act as a solvent but was removed from the liquid phase and thus also from the field of action as far as the freezing-point lowering is concerned. Under this assumption the unusual results obtained could be easily explained. Thus, if a clay, for example, causes

[1] Reference is made by number to "Literature cited," p. 217.

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15 per cent of the water to become inactive or unfree either physically or chemically, or both, and at 39 per cent of moisture this clay gives a depression of 0.075° and at 22 per cent, 0.987° C., then in the first case there is 24 per cent of moisture free or active to dissolve the salts, while in the second case there is only 7 per cent of free or active water for the same purpose. The depression of the freezing point at the low moisture content therefore would be many times greater than that at the high than would be expected from the total percentage of water content.

It was also found that the magnitude of the lowering of the freezing point of soils at the low moisture content decreased with successive freezings. This was true, however, only with complex and colloidal types of soil, such as the silts, loams, and clays, and not with the simple and noncolloidal soils, such as the sands and light sandy loams.

In explanation of this phenomenon the hypothesis was offered that a large portion of the water which was made inactive or unfree and thus removed from the field of action as far as the freezing-point lowering is concerned, was due to the colloids which the soils contain. This inactive or unfree water existed in the colloids both as physically adsorbed and loosely chemically combined water. Upon freezing, these colloids coagulated, the bonds uniting them with the water broke, and the combined or unfree water became liberated. This liberated and free water went to dilute the original solution and thus decreased the lowering of the freezing point. Thus, for instance, if there were 5 per cent of moisture at the beginning of the first freezing, there were probably 7 per cent at the end of the first freezing, 7.5 per cent at the end of the second freezing, and so on, until all the colloids were coagulated.

It was further discovered in these researches that solidification could not be induced to take place below a certain minimum moisture content, but it could be started extremely easily above this critical point. This minimum water content varied with the type of soil and appeared to correspond quite closely to the moisture content at which plants begin to wilt, or the wilting coefficient of soils. Since the percentage of moisture in many of the soils was quite high, amounting in some clays to 20 per cent and in some humus clays to 40 per cent and yet solidification could not be started, it appeared logical to conclude that the remaining water in the soil did not exist in a free state but in an inactive or unfree condition.

All evidences therefore seemed overwhelmingly in favor of the view that soils cause some of their moisture to become inactive or unfree and thus lose its solvent action and be removed from the field of action, so far as the freezing-point lowering is concerned.

The desire now arose to measure quantitatively the amount of this water which the different soils cause to become inactive or unfree and lose its solvent action. Such a determination, it was thought, would be of considerable scientific and practical importance.

After a thorough consideration it became apparent that the freezing-point method could not give a quantitative estimation of this inactive or unfree water without having to follow a rather complicated procedure. It could show only qualitatively that soils do cause water to become inactive and that the amount of this inactive water varied with the different soils. A search therefore was instituted to find some method which was simple, rapid, and accurate, and which could determine quantitatively the amount of water that the various soils cause to become inactive. After a considerable search the dilatometer method was chosen as the most promising for accomplishing the object.

The dilatometer method is an old one. The writer used it in the physical-chemical laboratory of Prof. G. Tamman at Göttingen University in 1913. Its employment, however, in the present study received the impetus from the work of Foote and Saxton (5), of Yale University.

It is an interesting coincidence that this work of Foote and Saxton, which really deals with the different forms of water held by the various hydrogels, appeared in March, 1916, while the writer's work (2), which contains the evidences bearing upon the ability of soils to cause water to become inactive, appeared in December, 1915, or only three months earlier. It will be of interest to mention that Foote and Saxton, using the dilatometer method, and the writer the freezing-point method, arrived independently at the conclusion that a considerable amount of water in certain solid substances refused to freeze, and consequently must exist in an inactive or combined form.

Although the idea of employing the dilatometer method to attain the writer's object received its impetus from the work of Foote and Saxton, the procedure followed and the form of apparatus used were devised in the course of the investigation and are entirely original and very different. As a matter of fact, Foote and Saxton give no description of the apparatus they employed, merely saying, "The dilatometer had the usual form."

PRINCIPLE OF THE METHOD

The principle of the dilatometer method as employed in the present investigation is based upon the fact that water expands upon freezing. If the amount of expansion that a certain quantity of water, 1 gm., produces upon freezing is known, then the amount of water that freezes in the soil can be calculated from the magnitude of expansion produced. If, also, the total water content in the soil is known, the amount that does not freeze can be ascertained by the difference.

DESCRIPTION OF THE METHOD AND PROCEDURE

A large amount of time was spent in endeavoring to devise a dilatometer which would be simple and give accurate and concordant results. A large number of dilatometers of different forms were tried; but they were

all discarded, and the one shown in figure 1 was finally adopted and employed throughout this investigation. It consists of a glass bulb, A, in which the soil is placed, the glass stem, B, on which the expansion is read, and the thermometer, C, which serves to indicate the temperature of the soil or mass and also acts as a stopcock to the bulb A. The bulb has a capacity of 50 c. c.; the stem is a 1 c. c. pipette and is calibrated to 0.1 c. c.; and the thermometer reads from $-5°$ C. to $+30°$, and is calibrated to 1°. That portion of the thermometer which is inserted into the mouth of the bulb is suitably ground down so that it forms water-tight connections with the latter.

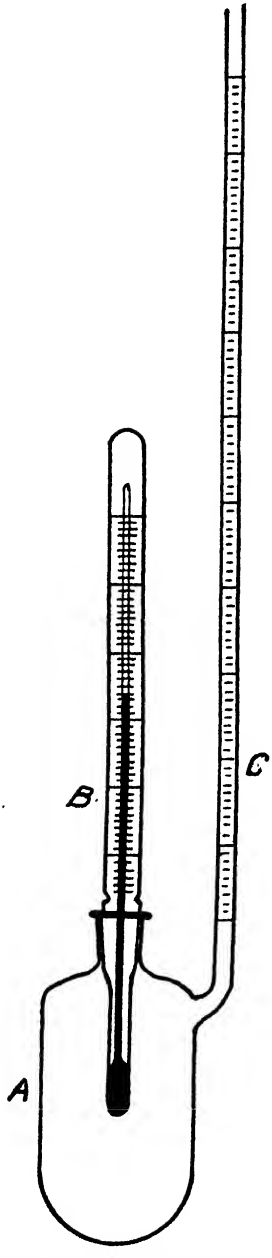

FIG. 1.—The dilatometer

It was found absolutely necessary to have a thermometer in the dilatometer, in order to indicate the degree of supercooling, because the amount of water that freezes in the soil is influenced to a considerable extent by the degree of supercooling. If the magnitude of supercooling was not the same, no concordant results could be obtained in the different samples of the same kind of soil.

For completely filling the bulb of the dilatometer after the soil was added and for measuring the volume of expansion of the soil water upon freezing, ligroin was used. Ligroin, of course, is a derivative of petroleum and is sometimes called "light petroleum." It proved to be a very satisfactory material for completely filling the bulb and for measuring expansion.

For determining the amount of water that soils cause to become inactive or unfree, the following procedure was adopted: To 25 gm. of air-dry soil were added 5 c. c. of distilled water. These were thoroughly mixed. In those soils which possessed a high water-holding capacity the mixing was done in an evaporating dish before the soil sample was placed in the dilatometer, while in those soils which possessed a low water-holding capacity the soil sample was first placed in the dilatometer and then the water added to it. In both of these processes precaution was taken to guard against the loss of water through evaporation and thus to avoid inequalities.

After the soil was placed in the bulb, the latter was completely filled with the ligroin. For expelling the air the soil was stirred with a rod until bubbles ceased to appear at the top. The process of stirring proved quite efficient in facilitating the removal of air.

After all the air was expelled from the soil, the thermometer was inserted into the mouth of the dilatometer and a sufficient amount of ligroin was placed in the stem, so that when the mass in the bulb had cooled, the column of the ligroin would be at the lower part of the graduated portion of the stem. The stem was then covered by a special cape in order to prevent the loss of the ligroin through volatilization.

The bulb of the dilatometer was then placed in a cooling mixture and allowed to cool. The cooling mixture consisted of crushed ice and common salt and was contained in a bath composed of two earthenware jars placed one inside the other and well insulated. The temperature of the cooling mixture was maintained at $-4°$ C. The dilatometer was allowed to remain undisturbed until the contents had attained the temperature of $-3°$, that is, supercooled to $3°$; and then by taking hold of the dilatometer by the stem it was gently moved in the cooling mixture until solidification began. The beginning of the solidification was indicated by the rise of the ligroin in the stem. The bulb of the dilatometer was allowed to remain in the cooling mixture, with a frequent movement until the rise of the liquid in the stem ceased. The time required for equilibrium to be attained varied with the type of soil, but it generally took about half an hour. This length of time, however, could be considerably reduced by moving the bulb in the ice mixture very often. The total rise of the ligroin in the stem was taken to represent the total amount of expansion due to the formation of ice.

Whenever it was desired to study the effect of successive freezings upon the amount of water that freezes, the bulb of the dilatometer was taken out of the ice mixture, wiped dry, and held between the hands until the soil was thawed, as indicated by the rise of the temperature on the thermometer; then it was placed back into the cooling mixture and the process described above was repeated.

For obtaining concordant results it was found essential to supercool the soil to the same degree. As will be shown subsequently, the amount of water that will freeze in a soil increases with the increase in supercooling. This is especially true of colloidal soils.

It was not found practicable to maintain in the bath a lower temperature below zero than $-4°$ C. If the temperature was lower than $-4°$, solidification would start before supercooling had taken place and no satisfactory results could be obtained on expansion. Furthermore, if the bath was too cold, the tendency for contraction would be too great, and the total expansion might not be indicated in the stem.

In performing tests to ascertain how closely duplicate determinations would agree by following the same procedure as closely as possible, it was found that in the majority of cases the duplicate determinations of the same kind of soil would agree very closely. Occasionally, however, the disagreement would be quite appreciable. This disagreement may be attributed partly to three factors.

In the first place, it appears that the phenomenon of solidification or crystallization does not start always the same; sometimes it starts with difficulty and its velocity is small, while at other times it starts very readily and its velocity is very high, even though the amount of supercooling in both cases is the same.

In the second place, the force of expansion may not be evenly distributed throughout the bulb, and consequently the entire expansion may not be indicated in the stem. It is perhaps due to this factor that stirring or moving the bulb in the cooling mixture hastens the equilibrium. This equilibrium would doubtless be attained in a shorter time, and the results would be much more accurate if the stirring was performed in the bulb. A dilatometer was devised in which the stirring could be performed in this manner. This dilatometer consisted of a long tube having a ground joint, and on this joint were attached the thermometer and the measuring stem. The contents of the tube were stirred by an electrical arrangement similar to that employed in the Beckman apparatus for performing freezing-point-lowering determinations. Unfortunately tubes with ground joints such as were necessary could not be obtained now in this country; and since it was necessary to have several of them, this form of dilatometer had to be abandoned.

In the third place, the water which the soils cause to become inactive or unfree is not in an absolute unchangeable condition, but is made free by various factors. Hence, if the empirical procedure of the method is not exactly the same, the duplicate determinations would naturally not agree.

In order to obtain the correct factor or value for converting the expansion due to ice formation into the corresponding weight, the expansion of 5 c. c. of distilled water was determined in the dilatometer employed. It was found that these 5 c. c. of water gave an expansion of about 0.5 c. c. upon freezing. According to these figures, 1 c. c. of water expands to about 0.1 c. c. upon freezing. This value is somewhat higher than that obtained by other investigators. Bunsen's data, for instance, show that 1 gm. of water at 0° C. expands 0.09070 c. c. upon freezing. In the computation of the data presented in this paper, however, the former value was employed. It is believed that for the writer's purpose this value is sufficiently correct; it is also very convenient.

EXPERIMENTAL WORK

QUANTITY OF WATER SOILS CAUSE TO BECOME UNFREE, AS INDICATED BY QUANTITY OF WATER THAT FAILS TO FREEZE

In accordance with the foregoing method, the amount of water that the soils cause to become unfree or inactive as indicated by the quantity of water that fails to freeze was determined. The soils employed varied both in type and origin. As to origin, many of the soils used had come from several different States. The results obtained are shown in Table I. The amount of water that fails to freeze is expressed both in cubic centimeters and in percentage of the 5 c. c. of water added to 25 gm. of soil. In every case the temperature of the bath was $-4°$ C., the soil was supercooled to 3°, and the readings are only of the first freezing.

TABLE I.—*Amount of water that fails to freeze in 5 c. c added to different soils*

State and name of soil	Water failing to freeze	Added water failing to freeze.	State and name of soil	Water failing to freeze.	Added water failing to freeze.
	C. c	*Per cent*		*C. c.*	*Per cent.*
Quartz sand.....	0 10	2.0	Florida		
Michigan.			Sand	0.50	10.0
Sand	.30	6.0	Do	.50	10.0
Sandy loam	.80	16.0	Do	.50	10.0
Do.	1 50	30 0	Do	.55	11.0
Do	1.15	30.0	Minnesota		
Silt loam	2.00	40.0	Sand	.50	10.0
Do	2.30	46.0	Sandy loam	1.50	30.0
Heavy silt loam	2 80	56 0	Clay.	4.00	80.0
Do.	3.10	62.0	Washington		
Do.	3 50	70.0	Sand	.50	10.0
Do	3.70	74.0	Fine sandy loam	1.15	23.0
Kentucky			Silt loam	1.65	33.0
Sandy loam	.90	18.0	Do	2.00	40.0
La Crosse sandy loam	1.15	23 0	Do	2.00	40 0
Parrington loam.	2.80	56.0	Heavy silt loam	2.20	44 0
Marshall silt loam	2.90	58.0	Wisconsin		
Texas			Plainfield sand.	.40	8.0
Sand	.20	5.0	Miami silt loam	1.40	28 0
Do	.30	6 0	Carrington silt loam	1.00	32.0
Fine sandy loam	.50	10 0	Colby silt loam	2 20	44.0
Sandy loam.	.90	18.0	Superior clay	3 50	70 0
Crawford clay	2 70	54.0	Pennsylvania		
Houston black clay.	3.00	60.0	Silt loam	1.40	28.0
			Do	1 50	30.0
			California		
			Clay loam	2.00	40.0

The results presented in Table I are really of great significance. They show that not all of the water added to the soils freezes; some of it refuses to freeze, and the amount that fails to freeze is entirely different in the various soils. It varies from 2 per cent in the case of quartz sand to 80 per cent in the case of Minnesota clay, of the 5 c. c. of water added to 25 gm. of soils. It increases, therefore, from the simple and noncolloidal types of soil to the complex and colloidal types.

If it is assumed that only free water freezes, then the water which fails to freeze is inactive or unfree, and has been transformed into this condition by the soils themselves.

RELATION BETWEEN THE AMOUNT OF WATER THAT FAILS TO FREEZE AND THE LOWERING OF THE FREEZING POINT

The amount of water that fails to freeze under the above empirical conditions bears a significant relation to the lowering of the freezing point. In Table II there are presented the percentage of water that fails to freeze and the value of the freezing-point lowering of a few typical soils. The depression was obtained by mixing 20 gm. of soil with 4 c. c. of water, thus making a ratio of 5 of soil to 1 of water. This ratio is exactly the same as that employed in the dilatometer determinations.

TABLE II.—*Amount of water that fails to freeze in 5 c. c. added to various soils and their corresponding lowering of the freezing point*

Name of soil.	Water failing to freeze.	Added water failing to freeze.	Lowering of the freezing point.
	C. c.	*Per cent.*	*°C.*
Quartz sand	0.1	2.0	0.009
Sand	.3	6.0	.074
Do	.4	8.0	.019
Do	.5	10.0	.018
Do	.5	10.0	.017
Do	.5	10.0	.011
Sandy loam	1.7	34.0	.138
Silt loam	2.0	40.0	.200
Superior clay	3.5	70.0	.392
Heavy silt loam	3.5	70.0	.312
Do	3.7	74.0	.440
Houston black clay	3.0	60.0	.422

An examination of the data of Table II reveals at once the significant fact that there is an exceedingly close relation between the amount of water that fails to freeze in the various soils and the degree of their freezing-point lowering. Those soils, for instance, which show the greatest lowering of the freezing point allowed the smallest percentage of water added to freeze, while those soils which indicate the smallest freezing-point depression permitted the largest percentage of water added to freeze.

The fact that not all of the added water freezes and the remarkable correlation between the percentage of unfrozen water and the lowering of the freezing point bear out the hypothesis proposed in former publications (2) that the abnormally high depression of soils at the low moisture content, or the abnormally great increase of the depression with the decrease in moisture content, is due to the ability of soils to cause water to become inactive or unfree and thus be removed from the field of action so far as the freezing-point lowering is concerned.

RELATION BETWEEN THE AMOUNT OF WATER THAT FAILS TO FREEZE AND THE WILTING COEFFICIENT OF SOILS

The amount of water that fails to freeze under the foregoing empirical conditions bears also a remarkable relation to the wilting coefficient of soils. In Table III there are presented the total percentage of water that fails to freeze and the wilting coefficient of some soils. The percentage of water that fails to freeze is expressed on the absolute dry basis, in order that it may be directly comparable to the wilting coefficient which is similarly expressed. The data on the wilting coefficient were obtained experimentally, following the mode of procedure described by Briggs and Shantz (4). Wheat was used as an indicator.

TABLE III.—*Relation between the percentage of water that fails to freeze and the wilting coefficient*

Name of soil.	Percentage of water that fails to freeze.	Wilting coefficient.
Sand	1.40	1.49
Sandy loam	3.52	4.28
Silt loam	10.50	9.62
Heavy silt loam	17.30	18.16
Do	15.86	13.82

A glance at Table III discloses at once the remarkable fact that the total percentage of water which does not freeze when the soil is supercooled to 3° in a temperature of −4° and the ratio of soil to water is 5 to 1, respectively, is the same as that at which plants begin to wilt, or the wilting coefficient. It will be observed that the variation does not exceed 2 per cent in any case.

RELATION BETWEEN THE AMOUNT OF WATER THAT FAILS TO FREEZE AND THE PERCENTAGE OF WATER AT WHICH SOLIDIFICATION REFUSES TO TAKE PLACE

As already mentioned, the investigations with the freezing-point method showed that solidification could be induced in all soils very easily from any maximum moisture content down to a minimum moisture content, and then it could not be started. It was shown in a former publication (2) that the percentage of water at which solidification could not be induced was the same as the wilting coefficient of soils, and it was suggested that the freezing-point method may be used to obtain the latter value. It may now be of interest to indicate that the percentage of water at which solidification refuses to start bears a close relation also to the amount of water which refuses to freeze in the dilatometer. This interesting correlation is exhibited in Table IV.

TABLE IV.—*Percentage of water that fails to freeze and the percentage of water content at which solidification refuses to take place*

Name of soil.	Percentage of water at which solidification refuses to take place.	Percentage of water that fails to freeze.
Sand	2. 10	1. 40
Sandy loam	5. 60	3. 52
Silt loam	10. 30	10. 50
Heavy silt loam	16. 00	17. 30
Heavy silt loam	13. 50	15. 86

It becomes at once evident from an examination of the foregoing results that the percentage of water at which solidification can not be induced to start and the percentage of water which fails to freeze are quite close in all the different types of soil.

RELATION BETWEEN THE AMOUNT OF WATER THAT FAILS TO FREEZE AND THE THERMAL CRITICAL MOISTURE CONTENT

In investigating the effect of temperature on some of the most important physical processes in soils, the effect of temperature on the movement of water vapor and capillary moisture was also studied (3). It was found that when one-half of a column of soil of uniform moisture content was maintained at 20° and 40° C. and the other half at 0° for eight hours, the percentage of water moved from the warm to the cold soil increased in all the different types of soil with the rise in moisture content until a certain water content was reached and then it decreased with further increase in the moisture content. The results then plotted into a parabola. The percentage of moisture at which the maximum thermal translocation of water occurred was different in the various classes of soil, but the percentage of the maximum thermal translocation of water was about the same for all classes for any one of the temperature amplitudes employed. The percentage of moisture at which this maximum translocation occurred was designated as "thermal critical moisture content."

It is now of great interest to state that this thermal critical moisture content is practically the same as the percentage of water which refuses to freeze in the corresponding soils—that is to say, the percentage of water in the soil at which the maximum thermal translocation occurs is practically the same as the percentage of water which refuses to freeze. This significant correlation was tested in about 10 different soils, and in almost every case the relation was exceedingly close, the variation not exceeding more than 3 per cent water content in any soil.

SIGNIFICANCE OF THE AGREEMENT OF THE VARIOUS METHODS IN INDICATING THE SAME PERCENTAGE OF WATER AS THAT WHICH FAILS TO FREEZE

The agreement of the various methods of entirely different principle in giving practically the same percentage of moisture as that which fails to freeze under the empirical conditions already mentioned is of great significance. This remarkable agreement indicates that there is a critical point on the moisture curve or scale at which the condition of the soil moisture begins to change, and this change or transformation must be very marked. At this point the soil water appears to change from the free state to an inactive or unfree condition. Whether or not the point of transition is abrupt can not be said definitely, but many evidences indicate that it is quite sudden.

On the other hand, it must be stated that these various methods probably do not indicate the absolute initial point of commencement of the unfree water. It is almost certain that the degree of unfree water as shown by the various methods is somewhat below that of the total. This is due to the fact that the unfree water does not exist in an absolute unchangeable and unutilizable condition, but in a state in which it can be changed into free water by various factors. As a consequence, the methods are not absolute, but are empirical—that is to say, since this unfree water can be converted into free water by various factors, then the amount of unfree water that any methods indicate will depend to a considerable extent upon the empirical conditions employed in that method. Thus, for instance, as will be shown subsequently, the amount of water that fails to freeze decreases with the increase in supercooling; the amount of unfree water therefore diminishes with greater undercooling. Again, the wilting coefficient of soils is not absolutely definite, but varies to a considerable extent with the environmental conditions which affect the intensity or velocity of atmospheric evaporation.

The agreement of the foregoing methods, therefore, in indicating the same percentage of unfree water must be considered more as a remarkable coincidence rather than as indicating the absolute initial point of commencement of the unfree water and consequently the absolute total amount of this unfree water.

EFFECT OF SUCCESSIVE FREEZINGS UPON THE AMOUNT OF WATER THAT FAILS TO FREEZE IN SOILS

The work with the freezing-point method showed, as already mentioned, that the lowering of the freezing point of colloidal soils at low moisture content decreased with successive freezings up to a certain number of freezings and then it became constant. This diminution was interpreted to mean that the soils had caused some of the water to

become inactive or unfree, to lose its solvent action, and to be removed from the field of action so far as the freezing-point lowering is concerned, and that the process of freezing changed the physical condition of the soil whereby some of this inactive or unfree water was liberated and went to dilute the original soil solution and thus decreased the lowering of the freezing point.

In order to ascertain whether this hypothesis was correct, the effect of successive freezings upon the amount of water that would freeze in soils was studied in the dilatometer. The procedure consisted of re-freezing the same sample of soil in the dilatometer as was employed in the previous study. After the data of the first freezing were obtained, the dilatometer was taken out of the bath, its contents thawed, and the process of freezing was repeated a second time. Numerous trials showed that in the majority of cases equilibrium was attained in the second freezing—that is, very little, if any, more water froze after the second freezing. Many trials also showed that not very much more water would freeze if the soil was maintained at a temperature of $-12°$ C. for one hour after the first freezing, than by supercooling it to 3° at a temperature of $-4°$, as followed in the regular procedure. In all the determinations made in this study, therefore, the soil was refrozen a second time in the same manner as in the first—that is, supercooled to 3° in a cooling mixture of $-4°$. The data obtained are detailed in Table V. The amount of water that fails to freeze in the first and second freezings is expressed both in cubic centimeters and in percentage of the 5 c. c. of water added to 25 gm. of soil. The difference in the percentage of water that fails to freeze in the first and second freezings indicates the effect of successive freezings upon the amount of water that becomes free.

TABLE V.—*Effect of successive freezings upon the amount of water that fails to freeze when 5 c.c. are added to various soils*

Freezing period.	Water in quartz sand failing to freeze.		Water in sand failing to freeze.		Water in sandy loam failing to freeze.				Water in silt loam failing to freeze.		Water in Carrington loam failing to freeze.		Water in heavy silt loam failing to freeze.				Water in superior clay failing to freeze.	
					First test.		Second test.						First test.		Second test.			
	C.c.	%	C.c.	%	C.c.	%	C.c.	%	C.c.	%	C.c.	%	C.c.	%	C.c.	%	C.c.	%
First	0.1	2.0	0.3	6.0	0.8	16.0	1.15	23.0	2.00	40.0	2.80	56.0	3.70	74.0	3.50	70.0	3.50	70.0
Second	.1	2.0	.3	6.0	.8	16.0	1.15	23.0	1.65	33.0	2.20	44.0	2.75	55.0	3.05	61.0	3.00	60.0
Difference	.0	.0	.0	.0	.0	.0	.0	.0	.35	7.0	.60	12.0	.95	19.0	.45	9.0	.50	10.0

The foregoing data show that the amount of water which fails to freeze in the second freezing is practically the same as in the first in the case of sands and light sandy loams or noncolloidal soils, but in the silts, loams, clay loams, and clays, or complex and colloidal types it is considerably less in the second freezing than in the first. In other words, repeated freezings increase the amount of free water and decrease to a corresponding degree the quantity of unfree water in the case of complex and colloidal types of soil but not in the simple and noncolloidal types of soil. As will be observed, in some of the loams and clays about 19 per cent more water froze in the second than in the first freezing.

These results therefore demonstrate that successive freezings cause some of the unfree or inactive water to become free in some soils and not in others. They go to prove, therefore, the validity of the hypothesis already mentioned, that the diminution of the lowering of the freezing point of colloidal soils at low moisture content with successive freezings is due to the dilution of the initial soil solution brought about by the inactive water in the soil becoming free through the process of freezing.

It must have been already realized, however, that it is not only the freezing that causes the unfree or inactive water to become free but also the thawing. If it were due only to freezing, then all the water that would become free would have done so during the first freezing. Instead, a large amount of water becomes free after the soil is thawed and frozen a second time.

Finally, it must be stated that if 10 c. c. of water are added to the 25 gm. of soil instead of 5 c. c., as was done above, the amount of water that fails to freeze in the second freezing is practically the same as that in the first, even in the complex colloidal types of soil. These results confirm those obtained with the freezing-point method, which show that the degree of the lowering of the freezing point of all types of soil at very high moisture content remains quite constant with successive freezings.

EFFECT OF DEGREE OF SUPERCOOLING UPON THE AMOUNT OF WATER THAT FAILS TO FREEZE

The amount of water that fails to freeze is dependent to a considerable extent upon the degree of supercooling. This is, however, true only in the complex and colloidal soils and not in the simple noncolloidal soils. The data bearing upon this phase of the investigation are presented in Table VI. These data were obtained at two degrees of supercooling, 1° and 3° C., in a cooling mixture of $-4°$. For each degree of supercooling a new sample of soil was used.

From an inspection of the data in Table VI it becomes at once clearly evident that the amount of water that fails to freeze is considerably less when the soil is supercooled to 3° C. than when it is supercooled to 1°. This, however, is true only in the case of the complex types of soil, the silts, loams, clay loams, and clays, but not in the simple types of soil,

such as the quartz sand, sands, and sandy loams. In some of the clay loams and clays the amount of water that fails to freeze in the supercooling of 3° is about 18 per cent less than in the supercooling of 1°.

TABLE VI.—*Effect of supercooling upon the amount of water that fails to freeze when 5 c.c. are added to various soils*

Degree of supercooling.	Water in quartz sand failing to freeze.		Water in sand failing to freeze.		Water in silt loam failing to freeze.		Water in heavy silt loam failing to freeze.					
							First test.		Second test.		Third test.	
	C.c.	*P. ct*	*C.c.*	*P. ct.*	*C.c.*	*P. ct.*	*C.c.*	*P. ct.*	*C.c.*	*P. ct.*	*C.c.*	*P. ct.*
To 1° C	0.1	2.0	0.4	8.0	3.10	62.0	4.15	83.0	3.70	74.0	3.30	66.0
To 3° C	0.1	2.0	0.3	6.0	2.20	44.0	3.50	70.0	3.10	62.0	2.80	56.0

Attempts were made to obtain data at still greater supercoolings than 3° C.; but it was found that if the temperature of the bath was increased above −4° in order to obtain the greater supercoolings, the soil began to freeze when it was cooled only a few tenths of a degree below zero. The highest temperature that could be obtained at which premature solidification would not start was −4°. Hence, the greatest supercooling that was possible at this temperature was 4°. For rapidity of operation, however, the supercooling of 3° was employed.

The decrease in the amount of the water that fails to freeze in the colloidal soils with the increase of supercooling is highly significant. It indicates that supercooling lends a certain amount of energy to the force of crystallization which overcomes the forces of the soils that cause the water to become unfree, and liberates this water so it can freeze. This decrease in the amount of water that fails to freeze with an increase in the supercooling bears an analogy to the increased quantity of the solid solvent that will separate from the solution with the increase in the degree of supercooling. It is a well-known fact that the amount of pure solvent separating into its solid phase upon freezing is increased with the degree of supercooling, and thus concentrates the remaining solution.

The above results also go to bear out the statement already made that the unfree water does not exist in the soil in an unchangeable condition, but that it can be made free by various factors, and that the magnitude depends upon the empirical condition of the method employed for its determination.

EFFECT OF THE AMOUNT OF MOISTURE PRESENT UPON THE AMOUNT OF WATER THAT FAILS TO FREEZE

In the course of the development of the procedures of the dilatometer method it was noticed that the quantity of moisture present in the soil influenced somewhat the amount of water that failed to freeze. In order to obtain very definite data upon this subject a series of determinations was performed with soils at two different water contents, 5 and 10 c. c. of water in 25 gm. of soil. The data obtained are detailed in Table VII.

TABLE VII.—*Effect of degree of water content present upon the amount of water that fails to freeze*

Name of soil.	Quantity of water failing to freeze when 5 c. c. of water were added.	Quantity of water failing to freeze when 10 c. c. of water were added.
	C. c.	*C. c.*
Quartz sand	0. 10	0. 10
Sand	.30	.40
Sandy loam	1. 15	1. 00
Silt loam	2. 00	1. 60
Heavy silt loam	3. 10	2. 30
Do	3. 70	2. 50
Do	3. 50	2. 60
Do	2. 80	2. 20
Clay	4. 00	2. 80

There are two columns of data in Table VII, one showing the amount of water that failed to freeze when 5 c. c. of water were added to 25 gm. of soil and the other when 10 c. c. of water were added to the same quantity of soil. An examination of the results in these two columns shows at once that actually a greater amount of water failed to freeze when 5 c. c. of water were added than when 10 c. c. were added. This is true, however, only in the case of the complex colloidal types of soil and not in the simple noncolloidal types. Thus, in the case of one of the heavy silt loams the amount of water that failed to freeze when 5 c. c. were added is 3.5 c. c., while the amount that failed to freeze when 10 c. c were added is 2.6, or a difference of 0.9 c. c. It will be observed that in almost every soil of the complex colloidal classes, about 1 c. c. more water failed to freeze when 5 c. c. were added than when 10 c. c. were added. In the case of the simple noncolloidal classes of soil, such as the sands, that quantity of water that failed to freeze was the same in both the smaller and larger quantity of water added.

The foregoing data in the case of the colloidal soils are really very significant. They indicate that the absolute amount of inactive or unfree water is greater at low moisture content than at high. Evidently an excess of water diminishes the quantity of water that the colloidal soils cause to become inactive or unfree.

Just why the quantity of water that fails to freeze in the colloidal soils is greater at the low moisture content than at the high can not be explained at this time definitely. The following suggestion, however, may be offered: It is probably partly due to the fact that the total force of solidification is greater at the higher moisture content than at the lower, and consequently more water is freed or extracted from the soil in the former than in the latter. In short, the rôle of the relative masses present comes into play.

CONDITION OF THE UNFREE WATER IN THE SOILS

It has been seen, therefore, that there is a certain amount of water in soils which refuses to freeze, the exact amount varying with the degree of supercooling, number of times the soil is frozen, and degree of moisture present. Under certain empirical conditions—25 gm. of air-dry soil mixed with 5 c. c. of water supercooled to 3° C. in a temperature of −4°—the percentage of water, on the absolute dry basis, that remains unfrozen corresponds remarkably closely to the moisture content known as the wilting coefficient, to the water content at which solidification can not be induced, etc. In order to distinguish this water from the free water which freezes easily, is readily available to plants, etc., it is designated as "unfree" water.

The question now is, How does this unfree water exist in the soil? Does it exist as capillary water, physically adsorbed water, loosely chemically combined water, or in all forms?

It must be stated in advance that it is not definitely known in which form or forms this unfree water exists. The problem is very complex and exceedingly difficult of definite solution. It is, however, under special investigation, and it is hoped that a definite solution of it may be presented at a later date. However, it may be stated at this time that there are many evidences, both direct and indirect, which indicate quite strongly that most of this unfree water exists as physically adsorbed and loosely chemically combined, with the latter probably predominating. Capillary water, as ordinarily understood, is present in small quantities. It probably abounds mainly in the initial region of the unfree water. The physically adsorbed and loosely chemically combined water probably exists in the solid phase or as solid solution.

The following are some of the evidences which go to indicate that most of the unfree water exists in a physically adsorbed and loosely chemically combined condition and consequently in the solid phase or as solid solution:

(1) As it has already been stated, the studies on the lowering of the freezing point of soils showed that this value increased in all soils with the exception of quartz sand and some extreme types of sand at a far greater rate than the percentage of water decreased. In other words, the ratio of the freezing-point lowering and the percentage of water were not inversely proportional (approximately) as might be expected, save only in the quartz sand and some of the sands. Thus, a clay at 92.76 per cent of moisture gave a depression of 0.039° C. and at 39.28 per cent, 1.075; the ratio of the percentage of water at the two moisture contents is only 2.37, while that of the depression is 27.56.

This phenomenon was explained on the supposition that some of the water contained by the soils was either physically adsorbed or loosely chemically combined or both, in which event this portion of the water

was not free or active to act as a solvent but was removed from the liquid phase and consequently also from the field of action, so far as the freezing-point lowering is concerned. Under this assumption the results could be easily explained. Thus, for example, if a clay caused 15 per cent of water to become inactive or unfree, either physically or chemically, or both, and at 39 per cent of moisture this clay gave a depression of 0.075° C., and at 22 per cent, 0.987°, then in the first case there was 24 per cent of free and active water to dissolve the salts and take part in the freezing-point lowering, while in the second case there was only 7 per cent of free and active water for the same purpose. The depression of the freezing point at the low moisture content therefore would be many times greater than that at the high, than would be expected from the total percentage of moisture content.

(2) It was also found in these researches on the freezing-point lowering of soils that the magnitude of the depression decreased with successive freezings. This was true, however, only in the complex and colloidal types of soil such as the silts, loams, clay loams, and clays, and not in the simple and noncolloidal types such as the quartz sand, sands, and light sandy loams.

In explanation of this phenomenon the hypothesis was offered that a large portion of water which was made unfree or inactive and thus removed from the liquid phase was due to the colloids which the soils contained. This unfree or inactive water existed in the colloids both as physically adsorbed and loosely chemically combined. Upon freezing, these colloids coagulated and the bonds uniting them with the water broke and the combined or unfree water became liberated and free. This liberated and free water went to dilute the original soil solution and thus decreased the lowering of the freezing point. Thus, for instance, if there were 5 per cent of free water at the beginning of the first freezing, there were probably 7 per cent at the end of the first freezing, 7.5 per cent at the end of the second freezing, and so on until all the colloids were coagulated.

It might appear that the foregoing data do not prove that the unfree water exists as physically adsorbed and loosely chemically combined and probably in the solid phase. It could also exist as capillary and film water in the liquid phase. If it is in the liquid phase, why does it not take part in dissolving the salts like the free water does, and be in equilibrium with the latter, so that the freezing-point lowering would not follow such an abnormal course?

(3) The view that some of the unfree water in the soil may exist as physically adsorbed and loosely chemically combined probably in the solid phase is considerably strengthened by the results obtained by Jones (6, p. 238) on the freezing-point lowering of hydrates. Jones found that the different hydrates such as calcium chlorid, magnesium

bromid, aluminium chlorid, etc., yielded far greater depression of the freezing point than would be theoretically expected. Jones attempted to explain these abnormal results by assuming that these hydrates take up water, forming complex compounds with it, and thus remove it from the field of action, so far as the freezing-point lowering is concerned.

(4) The chemical composition of the soil itself would probably seem to presuppose that some of the water in the soil must be chemically combined, if some of our prevalent chemical knowledge is correct. The present status of our knowledge of the chemical composition of soils indicates that the soil contains many colloidal hydrates, such as aluminium, silica, iron, and magnesium, in simple or in complex combinations or zeolites. If these compounds exist in the hydrate form, then the water of hydration, according to our present conception, is probably chemically combined.[1] The amount of water that many of these hydrates take up and combine with it, probably chemically, is really very great, as will be seen from the following formulæ: $Al_2O_3 \cdot 38H_2O$, $SiO_2 \cdot 1.35H_2O$, $Fe_2O_3 \cdot 4.25H_2O$, $Na_2SO_4 \cdot 10H_2O$, $Na_2CO_3 \cdot 10H_2O$, $CaCl_2 \cdot 6H_2O$, etc. The force with which this water of hydration is held by the different hydrates varies considerably. Such hydrates as $Na_2SO_4 \cdot 10H_2O$ lose most of their water upon being exposed to the ordinary atmosphere, while others, such as $CaCl_2 \cdot 6H_2O$, will lose it only at elevated temperature. Furthermore, all the water in any hydrate is not held with the same force but with a different degree of force. This is shown by the fact that the same hydrate possesses different degrees of aqueous pressure at the various degrees of hydration or formula weights of water. Thus, copper sulphate in the form of $CuSO_4 \cdot 5H_2O$ exhibits an aqueous pressure of 47 mm., while in the form of $CuSO_2 \cdot H_2O$ it shows an aqueous pressure of only 4.5 mm., at a temperature of 50° C. That all the water in a hydrate is not held by the same force is further confirmed by experimental data, which show that the greatest portion of water of hydration of most hydrates is lost below the temperature of 100°, while the remainder is given off above this temperature.

(5) That some of the unfree water may exist in the soil as chemically combined is further indicated by the researches of Muntz and Gandechon (8) on the heat generated by dry soils upon being wetted. These investigators found that a large amount of heat was generated when the soils were wetted with water, but very little if any when they were brought in contact with benzene and toluene. They reasoned that, if the heat

[1] The vapor tension of the salt hydrates, such as the $CaCl_2 \cdot 6H_2O$, $Na_2SO_4 \cdot 10H_2O$, etc., behaves quite different from that of the colloidal hydrates, such as the $Al_2O_3 \cdot 38H_2O$, $SiO_2 \cdot 1.35H_2O$, etc. In the former hydrates the vapor tension decreases by sudden steps as water is being withdrawn, indicating that definite hydrates are being formed. In the latter hydrates, however, the vapor tension decreases continuously without any sudden break in the curve, indicating probably that no definite hydrates are being formed. As a result of this difference in the vapor tension of the two classes of hydrates some investigators are led to believe that the water in the colloidal hydrates exists as physically adsorbed water and not as loosely chemically combined water. The opposite view is held by other investigators. Apparently the subject has not been definitely solved one way or the other. The problem is exceedingly difficult of definite solution.

generated is due to physical phenomena only, then the amount of heat produced must have been the same in the benzene and toluene as that in the water. They also found that soils would extract the water from alcohol. From all these data they concluded, although not positively, that water must be chemically combined in soils.

(6) In the present investigation it was found that if those soils which cause a large amount of water to become unfree, as indicated by the quantity that fails to freeze, are heated to red heat, they cause then very little if any water to become unfree. Indeed, they act almost like quartz sand. Their power to render water inactive, therefore, is destroyed by the process of heating.

These results could be interpreted to mean that these soils contain colloids, that it is these colloids which render the water inactive by combining with it physically and chemically to form colloidal hydrates, and that these colloids are destroyed by heating and are converted into an irreversible condition so that they can not take up the water to combine with it physically and chemically and assume the hydrated state again.

All the foregoing evidences therefore appear to indicate quite strongly that some of the unfree water exists in the soils as physically adsorbed and chemically combined.

In view of the presence of the colloidal hydrates in the soils and in view of the tremendous amounts of water that many of these colloidal hydrates take up, it also appears that the greater portion of the unfree water in the soils and especially in the colloidal types is chemically combined rather than physically adsorbed, provided the water in the colloidal hydrates of alumina, silica, and iron exists as loosely chemically combined water and not as adsorbed water.

The question now is, if this chemically combined and physically adsorbed water exists in the solid phase or as solid solution, can it be utilized by plants? The answer to this question may be obtained from the following facts: (1) This chemically combined and physically adsorbed water, although probably in the solid phase, is not in an absolutely unchangeable and unutilizable condition; it will evaporate almost like ordinary liquid water; it will become free by certain treatments; and it will be adsorbed by solid substances which exert an attraction for it, etc. (2) It has been found that plants will obtain water from ice (7), which is a solid solution. (3) In conducting soil-moisture studies Alway (1) and other investigators have found that plants under certain conditions can reduce the moisture content in the soil near the hygroscopic moisture content, and consequently below the wilting coefficient.

From these considerations, therefore, it appears that plants can and do utilize this unfree water which is conceived to exist as physically adsorbed and loosely chemically combined, probably in the solid phase.

On the other hand, the water in this condition must be held in the soil with a considerable force and the plants can obtain it only at an

exceedingly slow rate. If the intensity of the atmospheric evaporation is greater than the velocity with which the water is extracted from the soil and absorbed, the plants will wilt. The wilting of plants, therefore, appears to be due mainly to two factors: (1) To the large force with which the unfree water is held in the soil, and (2) to the great osmotic pressure of the soil solution at and below the wilting coefficient. As has been shown in other publications (2), the osmotic pressure of the soil solution of the complex types of soil slightly above the wilting coefficient amounts to 15 atmospheres, and its magnitude increases tremendously with small decreases in the moisture content.

The foregoing views that the soil moisture at the lower magnitudes of water content existing in the solid phase is due to the chemical and physical absorbing forces, and that the wilting of plants is due mainly to the great force with which this water is held in the soil and also to the large osmotic pressure of the soil solution, are contrary to the prevalent views upon the subjects. In the first place, it is quite generally believed that the water in the soil at the lower magnitude of moisture content and even below the hygroscopic coefficient exists as films in the liquid phase. That this is the common view is indicated by the following facts: (1) The movement of moisture is attributed to the curvature of the capillary films. (2) Attempts have been made to measure the thickness of these films around the soil particles or floccules; the crumb structure in the soil is attributed to the force of the water films, etc.

In the second place, the wilting of plants is attributed by some investigators mainly to the slow movement of the moisture in the soil. This view is well expressed by Shull (9) in a recent article. He says (p. 28):

> The view is held, therefore, that the wilting at this critical soil moisture content must be due to the increasing slowness of the water movement from soil particle to soil particle, and from these to the root hairs, the rate of movement falling below that necessary to maintain turgidity of the cells of the aerial parts, even under conditions of low transpiration.

Shull came to this conclusion from a comparison between the force with which the moisture is held in the soil at the wilting coefficient and the osmotic pressure of the sap or root cells. He measured the force with which the soils retain moisture by means of Xanthium seeds. He found that at the wilting coefficient this force amounts to only four atmospheres. The osmotic pressure of the sap of root cells amounts to about seven or eight atmospheres or twice as much as the force of the soil. From these data he logically concluded, therefore, that at the wilting coefficient there is still plenty of moisture and a gradient for the movement of water toward the plant, and yet the plants wilt; this wilting, therefore, is due to the slow movement of the soil moisture.

The magnitude of the force with which the moisture is held in the soil at the wilting coefficient, according to the method of Shull, is not confirmed by the results obtained by the freezing-point method and the

dilatometer. As has already been stated, the osmotic pressure of the soil solution alone is about 15 atmospheres, slightly above the wilting coefficient. Now, if the greatest portion of the moisture at this point exists as physically adsorbed and loosely chemically combined water probably in the solid phase, then this water must be held in the soil with considerable force, and this force must be added to that of the solution alone. The total force with which the moisture is held back in the soil, or the "back pull," according to these estimations, is many times greater than that obtained by Shull.

If, however, the wilting of plants is due to the slow movement of water, then why does not this water freeze if it is in the liquid phase and in an available form, since the rate of movement of water does not affect the freezing as it might the wilting of plants?

VALUE OF THE DILATOMETER METHOD

The functions of the dilatometer method may be summarized as follows: The greatest value of the method appears to be (1) in showing that soils cause water to become unfree or inactive, as indicated by its refusal to freeze; (2) in fixing an approximate estimation of the quantity of water thus becoming unfree; (3) in obtaining, under certain conditions, the wilting coefficient of soils very rapidly; and (4) in classifying, under certain arbitrary conditions, the water in the soil into free, capillary, physically adsorbed, and loosely chemically combined. This method promises to yield future results which will probably revolutionize our present knowledge concerning the moisture in the soil.

In all these functions, therefore, the dilatometer method appears to be of considerable value and importance in yielding knowledge on the fundamental questions regarding the effect of the soil upon the soil moisture and the condition in which the moisture exists in the soil.

SUMMARY

In the present paper there is presented the dilatometer method as a means of showing the amount of water which the soils cause to become unfree or inactive, as indicated by the quantity that fails to freeze.

The principle of the dilatometer method is based upon the fact that water expands upon freezing. If the amount of expansion that a given quantity of water, 1 gm., produces upon freezing is known, the total amount of water that freezes in a soil can be calculated. If also the total water content of the soil is known, the amount of water that does not freeze can be obtained by difference.

The dilatometer (fig. 1) consists of three parts: (1) a bulb, (2) a thermometer, and (3) a measuring stem.

The method of procedure consists of mixing soil and water in certain definite proportions, placing this moist soil in the bulb, and then filling

the latter with ligroin. The bulb with its contents is then placed in a cooling mixture and allowed to supercool. After the desired supercooling is attained the bulb is moved gently in the cooling mixture until solidification commences, which is indicated by the rise of the ligroin in the stem. The bulb is allowed to remain in the ice mixture with frequent movement until equilibrium is reached. The total rise of the ligroin in the stem is taken to represent the total quantity of water that freezes in the soil.

It was found that not all of the water added to soils freezes, some of it fails to freeze, and the quantity that fails to freeze is different in the various classes of soil. Under the empirical conditions of 25 gm. of air-dry soil mixed with 5 c. c. of water, supercooled to 3° C. in a temperature of −4°, the quantity that fails to freeze varies from 2 per cent in quartz sand to 80 per cent in clay, of the 5 c. c. of water added. It increases, therefore, from the simple and noncolloidal types to the complex and colloidal types of soil.

In the case of colloidal soils the amount of water that fails to freeze decreases with the increase in supercooling, but in the case of noncolloidal soils it remains the same.

By increasing the degree of moisture content the amount of water that fails to freeze is decreased in the colloidal soils, but remains practically the same in the noncolloidal soils.

At the low-moisture content successive freezings diminish the quantity of the unfrozen water in the case of the colloidal soils, but not in the noncolloidal soils.

The percentage of water content that fails to freeze in all soils under the empirical conditions of 25 gm. of air-dry soil mixed with 5 c. c. of water, supercooled to 3° in a temperature of −4° corresponds remarkably closely to the moisture content known as the wilting coefficient, to the percentage of moisture at which solidification can not be started, to the thermal critical moisture content, etc.

This water which fails to freeze is designated as unfree or inactive water. Its exact condition in the soil is not definitely known. There are many evidences, however, which indicate that a large portion of it may exist as physically adsorbed and loosely chemically combined, probably in the solid phase, or as solid solution. The quantity of the chemically combined probably exceeds that of the physically adsorbed.

This physically adsorbed and chemically combined water, although probably in the solid phase, is not in an absolute unchangeable condition, but it can be converted into free or available water, by various factors or treatments. Its magnitude, therefore, is not absolutely fixed, but varies with the empirical conditions of the method employed for its determination.

The wilting of plants appears to be due mainly to two causes: First, to the great force with which the moisture is held by the soil, and, second, to the large osmotic pressure of the soil solution at the wilting coefficient.

The dilatometer method appears to be of considerable value in showing (1) that soils cause water to become unfree, as indicated by its refusal to freeze, (2) in measuring quantitatively the amount of water thus becoming unfree, (3) in determining, under certain empirical conditions, the wilting coefficient of soils, and (4) in classifying, under certain empirical conditions, the water in the soil into free, capillary, physically adsorbed, and chemically combined.

The work herein reported should be considered only as preliminary. The investigations upon the subject are being continued.

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LEAFSPOT-ROT OF POND LILIES CAUSED BY HELICOSPORIUM NYMPHAEARUM

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INTRODUCTION

About the middle of May, 1913, the attention of this laboratory was drawn to an irregular spotting and decaying of leaves of pond lilies (*Nymphaea* spp.) in the aquatic gardens at Kenilworth, D. C. The disease was first noticed in the greenhouse propagating tanks, where some of the tender lilies were being started prior to setting in the open ponds. In some cases all the leaves succumbed, so that plants thus affected were too weakened for profitable growth during the following summer. As the season advanced, the disease appeared also in the open ponds, upon both the tender and the hardy varieties of pond lilies, and the inroads upon the leaves were so severe as to demand an attempt at control. From the general appearance of the leaf injury and its rapidity of spread from affected leaves it seemed evident that the disease was of a parasitic nature. On account of the severity of the disease in this particular locality and season, the present study was undertaken primarily to test the efficacy of spraying the floating leaves of a water plant with ordinary fungicides. However, the causal fungus itself proved so interesting that considerable time has also been devoted to a study of its characters and relation to the host. As demonstrated by similar signs on the host, isolation of the same fungus, and its successful inoculation into healthy pond-lily leaves, this disease has also been found by the writer at Arlington, N. J., at the Brooklyn Botanical Gardens, Brooklyn, N. Y., and at the New York Botanical Garden, New York City.[1] An apparently identical leaf disease was likewise seen at Riverton, N. J., though no cultures were attempted from this locality. Hitherto, so far as ascertained, no data upon this disease have been published.

SIGNS OF THE DISEASE

The disease first appears in the form of tiny dark specks on the leaf blade. (Pl. 67, *A*). In those varieties with red pigment in the cells of the lower surface, the initial specks are often reddish or bordered with red, as seen from the upper surfaces of the leaves. At first roundish in outline, the spots become more or less irregular with an increase in size until, either individually or by the coalescence of several initial infection

[1] The fungus from the New York Botanical Garden was isolated by Mrs. Ella M. A. Enlows, of the Bureau of Plant Industry.

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areas, they may, in the thinner leaved species, involve the entire leaf. (Pl. 68, *A*, *B*.) These areas are olivaceous black,[1] often somewhat lighter colored in the center, and they present a water-logged appearance. Although often present at first, the reddish border usually disappears with the rapid inroads upon the leaf made by the later stages. Finally in the thin-leaved species the entire blade may become a dark, olivaceous-black sodden mass of tissue which falls apart at the slightest touch. In the thick-leaved species, and especially in the case of older leaves, the spots, after attaining a diameter of 15 to 20 mm., may show no further increase for a considerable time. Such leaves, however, usually succumb in the end. Under conditions favorable for the parasite, the length of life of the host depends only on its capacity repeatedly to send up new leaves.

ISOLATION OF THE CAUSAL FUNGUS

In order to ascertain the nature of the parasite, several leaves of the pond lily (*Nymphaea odorata*) with initial stages of infection were collected from the open ponds at Kenilworth, D. C., in May, 1913. A few of these leaves were washed for three minutes in a 1 to1,000 solution of mercuric chlorid to kill surface organisms, so far as possible. They were then given three washings in sterile distilled water and placed in damp chambers. Another lot of leaves was placed directly in damp chambers without the previous sterilizing and washing. Freehand sections of young leafspots were examined under the microscope and showed sparingly a brownish, septate mycelium in the diseased leaf tissues. The leaves placed directly in damp chambers without washing developed various mold fungi, such as species of Penicillium and Botrytis, which soon covered the surface with a sporulating mass of hyphæ. Although these fungi were evidently saprophytic surface organisms which were outgrowing the real parasite, pure cultures were started for inoculation tests. In the meantime, after four or five days, signs of a fruiting fungus were noted on many of the leaves sterilized before being placed in damp chambers. Small tawny and somewhat powdery areas were seen on the surface of some of the leaf spots, and a microscopical examination showed these small masses to consist of large, many-septate, curved spores borne in clusters. These conidia were larger at the basal end, tapering and more or less helicoid at the apical end. This form alone appeared on the majority of the sporulating spots and gave promise of a causal relation. Corn-meal-agar plate cultures were poured, using these conidia and also using small pieces of leaf tissue from the younger spots. From the conidia single spore cultures were started by locating germinating conidia in the agar plates and transferring these to other plates of solidified sterile corn-meal agar, where the growth could be followed under the microscope. Colonies developing from these single spores were designated as isolation

[1] Ridgway, Robert, Color Standards and Color Nomenclature. 43 p., 53 col. pl. Washington, D. C., 1912.

205. The same fungus was also obtained in pure culture from pieces of the diseased leaf tissue. Three species of bacteria, one giving yellow and two giving white colonies, a sterile nonseptate fungus, and species of Aspergillus, Penicillium, and Botrytis were also obtained in culture by this method.

July 20–22, 1914.—Leaves of *N. odorata* with early stages of infection were collected in an open pond at the Brooklyn Botanical Gardens, Brooklyn, N. Y.; and two days later similar specimens were collected at Arlington, N. J. These were taken to the laboratory and plate cultures started from bits of diseased leaf tissue after sterilizing and washing the surface of the leaves. In this way a fungus similar to isolation 205 was obtained from each set of material. The fungus obtained from the Arlington material was designated as isolation 217 and that from the Brooklyn material as isolation 220.

August 24, 1914.—Affected leaves of one of the blue lilies were collected from an open pond at Kenilworth, D. C. Isolation by the method described in the preceding paragraph gave a fungus similar to isolation 205, which was designated as isolation 225.

October 30, 1914.—In the same way this fungus was reisolated from affected leaves of *N. odorata* inoculated on October 24, 1914, from a pure culture of isolation 225. This reisolation was designated as isolation 249.

The fungus was isolated in the same way by Mrs. Ella M. A. Enlows from affected leaves of *N. odorata* collected at Kenilworth, D. C. (Isolation En 59, September 6, 1915); and from similar leaves collected at the New York Botanical Garden, New York City (Isolation En 172, July 25, 1916).

The fungus was most readily isolated from young spots from the thicker leaved species of pond lily. In those with thin leaves various accompanying fungi and bacteria were more abundant, due, probably, to the more rapid softening of the tissues and earlier entrance of saprophytes.

INOCULATION EXPERIMENTS

Inoculation tests were made with all these isolations upon healthy leaves of both hardy and tender species of water lily. A part of these tests was made in the laboratory on cut leaves floated in tap water. Tiny spore clusters from corn-meal-agar cultures were scattered about in drops of water on the upper surface of each leaf without abrasion of the epidermis and the glass containers were left covered for a few days to give a moist condition for the germination of the conidia. Under these conditions the leaves are nearly in their normal environment except that the leaf petioles are severed from the parent plant. When not inoculated, these leaves have remained green and turgid for 10 days to 2 weeks or longer.

Unless otherwise stated, leaves for laboratory inoculation were collected from the open ponds at Kenilworth, D. C. Conidia used were from 1- to 2-weeks-old corn-meal-agar cultures, and the 10-inch glass

containers in which the leaves were floated were left covered for three days after inoculation. Control leaves were treated like the inoculated leaves, except that the fungus was not introduced.

Greenhouse inoculations were made upon plants growing in galvanized-iron tubs in one of the department greenhouses. These inoculations were carried out in the same way as in the laboratory. Each leaf was left covered for several days with a shallow bell jar supported on stilts so that the base of the bell jar dipped just below the surface of the water. Details of the inoculation experiments are given below.

LABORATORY, JUNE 14, 1913. ISOLATION 205.—Twelve leaves of *N. odorata* were inoculated on the upper surface, and four leaves, floated upside down, were inoculated on the morphologically lower surface. Four control leaves were not inoculated. At the same time two leaves each were inoculated with the following organisms isolated from diseased leaves: *Aspergillus* sp., *Penicillium* sp., *Botrytis* sp., a sterile nonseptate fungus, and the three types of bacteria. Two days later tiny dark-reddish specks were observed on the leaves in every case where the upper surface had been inoculated with isolation 205, except where parts of the surface of the leaves were several millimeters below the surface of the water. In the latter cases no signs of infection were evident. Leaves inoculated on the lower surfaces, those inoculated with other fungi and bacteria, and the control leaves showed no signs of infection throughout the experiment.

After four days the infected areas were 3 to 4 mm. in diameter, the centers brownish, and the outer parts dark olivaceous black. Deeply submerged parts of leaves were still unharmed by the presence of the fungus. The leaves inoculated on the lower surface were mostly submerged, and no visible infection had occurred, except at a point in one leaf near the surface of the water where a break had occurred in the epidermis. The experiment was run for 10 days with no further change except an increase in size of infection areas until at the close, when the infected leaves were for the most part a mass of decaying tissue. The tests with the other forms of fungi and bacteria were repeated twice and in every case gave a negative result, so that these organisms were discarded and attention devoted henceforth to the form represented by isolation 205.

LABORATORY, JUNE 19, 1914. ISOLATION 205.—Eight leaves of *N. odorata* were inoculated with this isolation. After two days infection had started at the inoculation points. After five days the spots were 3 to 35 mm. in diameter. Each inoculation had taken, and the three controls were perfectly sound.

LABORATORY, JULY 7, 1914. ISOLATION 205.—Inoculations were made on 12 leaves of *N. odorata*, 6 leaves of *N. caerulea* (Pl. 68, *A*, *B*), 6 leaves of *N. tuberosa* (Pl. 67, *A*), and 3 leaves of one of the tender day-blooming blue lilies. After three days tiny infection specks were observed at nearly every spot inoculated. After four days infection had started at all inoculation points. In most cases there were small reddish spots up to the size of a pin head. After a week single spots had increased in area up to 30 and 35 mm. in diameter. The main part of the diseased spots was water-soaked and olivaceous black in color, and in many cases the margins were reddish. No controls were run with this test.

LABORATORY, AUGUST 8, 1914. ISOLATIONS 205 AND 227.—One leaf each of *N. odorata* and *N. daubeniana* was inoculated with isolation 205, and one leaf of each species was held for a control. After four days small reddish spots were visible at the inoculation points on both leaves. After six days the spots had increased considerably in size, and were olivaceous black and water-logged. The controls were sound.

By the same method, inoculations with isolation 205 were also made on four healthy leaves of Egyptian lotus (*Nelumbium speciosum*). In this case the glass con-

tainers were left covered for five days, but after eight days no signs of infection had appeared.

A species of Alternaria obtained from a leafspot of lotus was tested upon four leaves of *N. odorata*. No infection had resulted after eight days.

LABORATORY, AUGUST 8, 1914.—Isolations 217 and 220 were inoculated on four leaves each of *N. odorata*. After four days small infection spots had started on all the inoculated leaves and at the majority of points of inoculation. After eight days the infections had developed into the typical irregular spots. The four control leaves were sound.

LABORATORY, OCTOBER 16, 1914. ISOLATIONS 205 AND 225.—Inoculations were made with isolation 205 on four leaves of *N. odorata*, and one of *N. daubeniana*, and with isolation 225 on three leaves of *N. odorata* and one of *N. zanzibariensis*. These inoculations were from 45-day-old corn-meal-agar cultures of each isolation. In 5 to 6 days all inoculations with 205 had taken. Two out of three leaves inoculated with 225 showed infection but the third, together with the leaf of *N. zanzibariensis*, remained healthy. Five leaves of *N. odorata* and one each of *N. daubeniana* and *N. zanzibariensis* held through the test as controls remained sound. In this case the fungus retained its infective power in culture after being held 45 days at laboratory temperature (20°–25° C.).

LABORATORY, OCTOBER 24, 1914. ISOLATIONS 205 AND 225.—Six leaves of *N. odorata* and three leaves of one of the night-blooming species of *Nymphaea* were inoculated on the upper surface with isolation 205, and two leaves of *N. odorata* with isolation 225. Four leaves of each species were floated upside down in the glass containers and inoculated with isolation 225.

After three days small reddish infection spots were starting at most of the inoculation points in all the leaves inoculated on the upper surface. After six days all these inoculations had taken, giving the typical spots, which were now up to 5 or 6 mm. in diameter. Most of the spots were surrounded by a halo of pale yellowish green tissue grading off to the normal green of the leaf. No fungus mycelium could be found in this outer halo. It seems thus evident that a substance or substances produced by the fungus may diffuse into the living leaf tissues and cause the death of the cells ahead of the actual fungus invasion.

After three days no infections were apparent on the inverted leaves. After five days infection areas were starting at inoculation points at the water surface or where the leaf epidermis was covered only by a very thin film of water. Inoculated portions covered by an appreciable depth of water (for example, several millimeters) still showed no signs of infection although in many cases the spore masses were still resting directly on the leaf epidermis. Infections thus took place where the lower epidermis was not submerged, but much more slowly than where inoculations were made on the upper epidermis.

The four control leaves run with these two sets of inoculations were and remained sound throughout.

On October 30, reisolation of the fungus was made from leaves inoculated October 24 with isolation 225. This reisolation was designated as isolation 249. Subsequent inoculations with this isolation gave typical infections.

GREENHOUSE, NOVEMBER 9, 1914. ISOLATIONS 205, 225, AND 249, USING 2- TO 4-WEEKS-OLD CULTURES.—Inoculations were made with isolation 205 on two upper leaf surfaces each of *N. omarana*, *N. rubra*, *N. dentata*, *N. odorata;* with isolation 225 on *N. rubra*, *N. zanzibariensis*, *N. omarana*, and *N. odorata;* and with isolation 249 on *N. daubeniana* and *N. capensis*. Two tubs containing *N. caerulea* and two containing *N. tuberosa* were retained as controls. All inoculated and control leaves were covered by shallow bell jars for four days after inoculation and in each species two to four leaves were inoculated. After three days evidences of infection were observed on many of the inoculated leaves. After five days every inoculated leaf showed the typical spots,

and, so far as could be seen, infection had occurred at every inoculation point. In the case of leaves with red pigment on the lower surface there was a tendency toward a slight reddish border around the spots as seen from the upper surface.

LABORATORY, NOVEMBER 10, 1914. ISOLATIONS 205, 225, AND 249.—Inoculations were made with isolation 205 on five leaves and with isolations 225 and 249 on two leaves each of *N. odorata*, collected from plants growing in one of the department greenhouses. Typical infection spots were visible in three days on all inoculated leaves. After four days infection areas were evident so far as could be determined at every inoculation point, and these varied in diameter from the size of a pin head up to 20 mm. in diameter. The five control leaves of the same species remained sound.

LABORATORY, DECEMBER 31, 1914. ISOLATIONS 205 AND 225.—Inoculations were made with each isolation on five leaves of *N. capensis* (Pl. 68, *C*) and five leaves were held as controls. These leaves were collected from plants growing in one of the department greenhouses. After four days infections were starting at every inoculation point and after one week the olivaceous-black spots varied from mere specks to spots 20 mm. in diameter. The lighter green halo surrounding the dead area, as noted under a previous test, was much in evidence here. This halo was most prominent in the thinner leaved species of pond lily. The five control leaves remained sound.

LABORATORY, APRIL 16, 1915 ISOLATION 205.—Four leaves of *N capensis*, one of *N. zanzibariensis*, and two of *N. caerulea* were inoculated from a single 2-months-old corn-meal-agar culture. After three days very numerous pinhead spots were observed on all three species of lily, and several olivaceous-black spots up to 5 mm. in diameter on *N. capensis*. After 5 days some of the leaves of *N. capensis* were a mass of water-soaked decaying tissue. Large water-soaked areas and numerous small spots occurred on leaves of the other two species. The light-green halo was noted around the decayed spots on all three species. The four control leaves showed no signs of infection except that one of them had a small spot at the beginning of the experiment and this continued to develop. Apparently it was a natural infection with the same fungus.

LABORATORY, JUNE 25, 1915. ISOLATION 225. INOCULATED BY MRS. ELLA M. ENLOWS.—One leaf each of *N. odorata*, and *N. zanzibariensis* was inoculated with this isolation. After three days small infection areas from mere specks up to 3 mm. in diameter were observed. The decay progressed rapidly so that after one week the two leaves were a mass of soft-rotted tissue. The three control leaves remained sound.

LABORATORY, NOVEMBER 17, 1915. ISOLATIONS 205, AND EN 59, USING 4-DAY-OLD CORN-MEAL-AGAR CULTURES.—Inoculations were made with 205 on one leaf each of *N. capensis* and *N. omarana;* and with En 59 on one leaf of *N. capensis* and two leaves of *N. omarana*. After two days tiny infection specks were noted at the points inoculated with En 59 on leaves of *N. omarana*. After four days infections had started on all leaves inoculated with each isolation, and after six days the olivaceous-black infection areas from En 59 had in many cases a diameter of 12 to 15 mm., and those from 205 in no case were more than 5 mm. in diameter. A larger number of infection areas had resulted and increase in size of spots was more rapid with isolation En 59 than with 205. Isolation 205 had been carried in culture for over two years, while En 59 was a fresh isolation. Possibly this may account for the difference in virulence.

LABORATORY, DECEMBER 13, 1915. ISOLATION EN 59.—Sclerotia from a 2-weeks-old corn-meal-agar slant were used for inoculating three leaves of *N. omarana* and conidia from the same lot of cultures for inoculating two leaves each of *N. zanzibariensis* and *N. omarana* and three leaves of *N. capensis*. After two days tiny infection spots were noted on the leaves inoculated with conidia, but none on those inoculated with sclerotia. After three days small infection spots were just visible on the three leaves inoculated with sclerotia, while they were as large as 2 mm. in diameter on the

conidial inoculations. Observation after nine days showed typical spots, some of which were 40 mm. in diameter, and so far as could be determined infection had occurred at all points of inoculation. The yellowish halo around the infection areas was prominent in each case.

PARASITISM OF THE FUNGUS

Through the inoculation tests above outlined the parasitism of this fungus to leaves of pond lily (*Nymphæa* spp.) is clearly established. Inoculations were made during three years with six different isolations obtained from four localities on leaves of nine species of pond lily. In one experiment where cultures 45 days old were used, seven only out of nine inoculated leaves developed the disease. However, all other inoculations, including several leaves inoculated from cultures 2 months old, were successful. Thus, out of a total of 135 leaves inoculated on the upper surface during three seasons, 133 leaves developed the disease in many places. In fact, in nearly all cases, so far as could be determined, the diseased spots appeared wherever the conidia were placed.

Inoculations on the lower epidermis of pond-lily leaves were partially successful. These infections when they occurred were slower in starting, but once started the progress of the disease was apparently as rapid as with inoculations from the upper surface.

In the one inoculation test made with this fungus on leaves of Egyptian lotus (*Nelumbium speciosum*) no infection resulted.

CULTURAL CHARACTERS OF THE FUNGUS

The gross characters of the fungus as grown upon several of the common culture media are as follows: Color descriptions are according to Ridgway.[1]

On beef-agar slants a smooth, pale, vinaceous fawn to vinaceous buff, feltlike mat of mycelium soon develops on the surface of the medium. After two to three weeks no further change takes place except a gradual drying of the agar. No sclerotia have been observed, but branching hyphæ with swollen cells are frequent, and somewhat atypical conidia are sparingly developed.

In beef bouillon in test tubes a water-tight felt is formed at the surface of the liquid, the latter remaining clear after several weeks. This mycelial mat is at first whitish but later approaches a pale vinaceous fawn to vinaceous buff color.

On corn-meal-agar slants the growth is at first whitish with scant aerial mycelium. In four to five days conidia begin to develop in the central portion of the colonies, giving a velvety appearance to the surface. In color the sporulating surface is Dresden brown to mummy-brown. After two to three weeks the dark, velvety sporulating portion nearly to quite covers the surface of the slant and the medium to the depth of several millimeters gradually assumes a light purplish-vinaceous to purplish-vinaceous cast which grades off into the pearl-gray of the agar. From this diffusion of the pigment into the medium it is clearly shown to be a water-soluble substance. Black, roundish sclerotia consisting of a solid mass of fungus cells often develop in old corn-meal-agar cultures (Pl. 67, *C–E*).

[1] Ridgway, Robert. Op. cit.

On cooked white corn meal in flasks the mycelial growth is feltlike with somewhat cottony, pale purplish vinaceous to light pinkish cinnamon aerial mycelium at the surface of the medium. The sporulating parts are a Dresden brown to mummy-brown, while the sclerotial masses in old cultures approach a deep neutral-gray. Conidia are produced rather sparingly, but rounded to irregular black carbonaceous sclerotia are produced abundantly after two to three weeks' growth.

In lavender-blue litmus milk in test tubes a surface felt is formed with no growth in the lower part of the tube. Around the edge of the tubes the felt is slightly paler than pale purplish vinaceous with cinnamon to orange-cinnamon rings; while over the upper surface it approaches Dresden brown to mummy-brown. The casein is gradually precipitated, leaving a dark purplish supernatant fluid. The part above this shows no change in color when compared with check tubes, but bluish rings finally develop around the margin of the surface growth

On oxalic-acid-agar slants the surface mycelium at first approaches light purplish vinaceous, later becoming fawn color. The sporulating surface is nearly avellaneous. Conidia are sparingly developed, but are not as uniform in size or shape as on corn-meal agar. The red color of the medium (due to addition of neutral-red) becomes gradually bleached out, until in three to four weeks it assumes the color of plain beef agar.

On potato-agar slants a Tilleul-buff cottony mycelium develops within a few days over the surface of the agar. A moderate number of typical conidia are borne on the finally somewhat feltlike mycelium. Even after two or three weeks there is no further increase in the number of spores borne. No sclerotia have been noted.

On cooked potato cylinders the appearance is somewhat similar to that on potato agar. The loose feltlike growth is Tilleul buff to vinaceous-buff in color. No conidia or sclerotia have been observed even after three to four weeks.

On cooked rice in test tubes a feltlike growth of shell-pink to buff-pink mycelium is formed. In four to seven days spores begin to develop, giving the surface a shade closely approaching Saccardo's umber. Conidia and sclerotia are fairly abundant, but the latter are not so regular in shape as on cooked string beans.

On cooked string beans the cottony mycelium covering the surface at first is a Tilleul buff varying slightly in shade on both sides of this color. The sporulating surface developed after four to ten days approximates a dark mouse-gray. Typical conidia are abundantly developed, and after one to three weeks there appear considerable numbers of roundish sclerotia. These have all the external appearances of pycnidia or perithecia, but on breaking upon a slide, or sectioning, these were invariably found to consist of a solid mass of fungous cells, and no spores of any kind were to be found.

On synthetic agar [1] slants a thin feltlike mycelial weft covers the surface in a few days. This weft corresponds almost exactly to the shell pink of Ridgway's chart.[2] After about three weeks' growth the agar, which is a very dark brown at first, becomes bleached to about the color of plain beef agar. No spores, sclerotia, or swollen cells are developed on this medium; but numerous round, highly refractive bodies occur in the hyphæ.

[1] (1) 1,500 c c. of distilled water and 36 gm. of agar. Cook in double boiler for one hour at 15 pounds' pressure.

(2) 500 c. c. of distilled water, 200 gm. of dextrose. 40 gm. of peptone, 20 gm. of ammonium nitrate, 5 gm. of magnesium sulphate (crystals), 10 gm. of potassium nitrate, 5 gm. of potassium acid phosphate (K_2HPO_4) and 0.2 gm. of sodium chlorid.

Boil in double boiler for 30 minutes, add agar, and cook for five minutes. Restore to volume, titrate, cool to 60° C., and add whites of two eggs. Cook to coagulate eggs; filter, tube, and sterilize

This formula is modified from that given by Darwin and Acton. (Darwin, Francis, and Acton, E. H. Practical Physiology of Plants. ed. 3, p. 68. Cambridge, [Eng.], 1901.)

[2] Ridgway, Robert. Op. cit.

VIABILITY OF THE FUNGUS IN CULTURE

In order to ascertain the viability of the fungus in culture under ordinary laboratory conditions transfers to corn-meal-agar slants were made from time to time during two years, and plates poured from old corn-meal-agar cultures. Tests were made of cultures 11 months old. None of this age were viable. With strains 205 and 225 three tests each were made at different times with cultures 3½, 4, 5, and 7 months old, respectively; and with 249, three tests with cultures 3½ months old, and one test each with cultures 5 and 7 months old. At the same time that transfers were made to corn-meal-agar slants, corn-meal-agar plates were poured from each of these old cultures so that germination could be followed under the microscope (Pl. 69, *A*). Up to seven months every culture tested gave growth readily on corn-meal-agar slants and in petri dishes, and the colonies developed the typical conidia in abundance. Examination of the petri-dish cultures showed that germination occurred from both conidia and sclerotia. Two successful sets of inoculations were made on pond-lily leaves directly from cultures 45 days and 60 days old, respectively. In addition, transfers from a few of the 3½, 4, 5, and 7 months old cultures were tested by inoculation on leaves of pond lily; and in each case typical infection resulted. This, together with microscopical examination, showed the colonies to be from the original fungus and not from an intruder. This would tend to show that the conidia and sclerotia are both capable of carrying the fungus over for a considerable period of time under certain adverse conditions, such as drying and increased acidity of the medium, at temperatures ranging from 20° to 30° C.

SCLEROTIAL GERMINATIONS

Sclerotia from 1-, 3-, 6-, 8-, 9-, and 11-months-old corn-meal-agar cultures were sown on sterile moistened sand and sterile moistened garden soil and held at laboratory temperature (about 20° C.). Germination took place from all the sclerotia, except in the case of those from the 11-months-old cultures, but microscopical examination at intervals during three weeks failed to show any sporulating form in connection with them. Sections after two and three weeks showed the sclerotia still made up of a solid mass of fungus cells, hyalin in the interior and brownish and thick-walled on the exterior. This is the structure shown by both young and old sclerotia in culture (Pl. 67, *C*, *E*), and in no case have evidences of a pycnidial or perithecial stage been observed.

Single sclerotia separated from mycelium and conidia and sown in fresh culture media readily send out germ tubes from the superficial cells (Pl. 67, *D*), and successful inoculations to leaves have been made, using such isolated sclerotia.

TEMPERATURE TESTS

Of 80 transfers of two isolations of the fungus to corn-meal-agar slants 40 were placed at once in a 10-compartment refrigerator with temperatures ranging from 2° to 19° C. The other half were placed in the compartments the following day, after the conidia had germinated. Where germination had occurred before placing cultures in the refrigerator, a very slight growth took place at 2°, but no perceptible growth occurred where transfers were held from the start at this temperature. Slight mycelial growth took place at 4° to 5° in all tubes with increase in rapidity following a rise in temperature. Development of conidia began at 8° to 9° and increased in rapidity up to the highest temperature tested. Sclerotia began to develop at 8° to 9°, increased in numbers up to 14° to 15°, and declined in numbers up to 19° to 20°, where but few were formed. All cultures held at the lower temperatures developed rapidly when later placed at 18° to 20°. These observations covered a period of five weeks.

HOST-PARASITE RELATION

Numerous microscope sections have been made of naturally infected leaves and also of artificially infected leaves at periods of time varying from a few hours to several days after inoculation. Study has also been made of portions of leaves killed, bleached, and stained without sectioning at 18, 24, 48, and 72 hours after sowing the conidia on the upper surface, as in the inoculation experiments previously detailed. In the older spots the tissues are seen to have more or less completely collapsed and fungus mycelia are found ramifying both between and within the decaying cells, together with coccoid and rod-shaped bacteria and various protozoa, a condition to be expected in a decaying mass of tissue floating on a watery medium. In the case of leaves studied without sectioning, germination of the conidia has been noted on the leaf surfaces at 18 and 24 hours after inoculation, and in several cases at 24 to 48 hours the germ tubes were seen entering a stomatal opening. By focusing down the continuation of the hyphæ was also seen below the guard cells and epidermal cells in the substomatal cavity.

Furthermore sections of leaf spots at three to four days after inoculation have shown in numerous instances the hyphæ entering the stomatal opening and branching out in the chamber below (Pl. 70, *B*, *C*). In slightly older spots the pale brownish hyphæ have repeatedly been seen ramifying between the cells of the spongy parenchyma and through the large air chambers, as also between the cells of the palisade tissue. But, except in old decayed and water-logged spots, hyphæ have not been seen within the cells.

It is clear that infection may and does occur through the stomata, but this is very evidently not the only method. Infection occurs,

although somewhat more slowly, when inverted leaves are inoculated on the morphologically lower surface, although stomata are present only on the upper surface.

The usual occurrence around the developing spot of a pale yellowish green halo grading off to the normal green of the leaf has been noted previously (Pl. 67, *A*; 68, *C*). No mycelium has been found in this bordering tissue; and even if sometimes present in the intercellular spaces, it is almost certainly not intracellular. From these facts it is evident that some product or products of the fungus metabolism are capable of diffusing into, injuring, and finally killing the host cells.

The epidermal cells in sections of healthy leaves stained with Flemming's triple stain (Pl. 70, *A*) are seen to be free from coagulation products, the nuclei are clearly differentiated, and the numerous chlorophyll bodies are well rounded out and definite in outline. In the infected areas even at so short a time as three to four days after inoculation the epidermal cells are frequently filled more or less completely with coagulation products, the nuclei are disintegrating, and the chlorophyll bodies are fewer in number, and those left are seen to be disintegrating (Pl. 70, *B*, *C*). The diseased parts of the leaf take the orange stain more readily, while the healthy parts show a decided affinity for the gentian violet. These differences can not be due to variations in individual leaves nor to differences in microtechnic, for where sections have been made through the edge of a young spot into normal tissues beyond it, all these differences have been seen in a single section.

Conidia have been found in one or two cases on freshly collected material and have developed twice on the upper surface of leaf spots in a damp chamber. Usually, however, after the removal of diseased leaves from the water and after they had been placed in a damp chamber, a fine whitish growth of more or less erect hyphæ develops over the infected areas without the formation of conidia. In several instances sclerotia have developed on the leaf spots after the leaves have been placed in a damp chamber.

The fact that infections have not been found to occur regularly on submerged leaves is probably due in part to a lack of sufficient oxygen, since in liquid and solid media growth of the fungus is almost entirely limited to the surface.

TAXONOMY AND DESCRIPTION OF FUNGUS

This pond-lily parasite belongs to the hyphomycetous group of the Fungi Imperfecti. It is to be included in the family Dematiaceae among those forms with dark, loose hyphæ and conidia, but without definite stromata or fruiting bodies. The elongated, transversely septate, more or less helicoid conidia place it in the genus Helicosporium belonging to the small group Helicosporae. Of the two other genera in this group, Helicopsis is without definite mycelium and Helicoma bears muriform conidia.

The following is a brief description of the fungus under discussion:

Helicosporium nymphaearum, n. sp.

Upon leaves of pond lilies (*Nymphaea* spp.), causing initial brown to reddish flecks, developing into irregular olivaceous-black water-soaked spots, which individually or by coalescence may in some cases involve the greater part of the leaf blade.

Mycelium light brown, intercellular, often hyalin in culture, septate, branched, growing readily on ordinary culture media.

Sporophores long and slender and bearing several conidia near the summit (Pl. 69, *D*). Conidia brown, many septate, constricted at the septa, usually somewhat larger toward the basal end, slightly tapering toward the curved or usually helicoid apical end (Pl. 69, *C*, *E*); outer walls thick and with echinulate sculpturings (Pl. 69, *D*, *F*, *G*); cross walls with roundish thin spot or perforation in the center (Pl. 69, *B*); highly variable in size (60 to 190 by 5 to 18μ); not readily produced on diseased leaves in damp chamber but develop in large numbers on several of the ordinary culture media. Sclerotia roundish, subcarbonaceous, develop sparingly on leaves and in culture, 150 to 900μ diameter.

Habitat: Leaves of *Nymphaea* spp. Type specimens from aquatic gardens at Kenilworth, D. C. Specimens also collected from open ponds at Riverton and Arlington, N. J., at the Brooklyn Botanical Gardens, Brooklyn, N. Y., and at the New York Botanical Garden, New York City.

Helicosporium nymphaearum, sp. n.—Maculis in foliis primo minutis fuscis vel subrufis, deinde subnigris, aquosis et irregularibus, interdum conjunctis postremo; mycelio albido-fusco, intercellulari, septato, ramoso; saepe in culturis hyalino, sporophoris longis, tenuibus, multas conidias prope apicem habientibus; conidiis fuscis, multiseptatis, in septis constrictis, in basi et apice plerumque attenuatis; apicibus conidiarum curvatis vel plerumque helicoideis; muris conidiarum densis, echinatis; conidiis 60–190μ × 5–18μ; Sclerotiis subsphaericis, subcarbonaceis 150–900μ.

Hab. in foliis vivis Nymphaeae speciorum, Washington, D. C, Arlington et Riverton, N. J., New York, N. Y. America Borealis.

CONTROL OF THE DISEASE

The control of diseases of aquatic plants has been little studied. In fact, the only account of actual experiments along this line which has come to the attention of the writer consists in a brief account of tests for the control of a leafspot of pond lilies [1] apparently due to a species of Cercospora. No cultural or inoculation experiments with the parasite are mentioned, but a description is given of a single series of experiments with Bordeaux mixture, oxid of copper mixed with sulphur, sulphur alone, and ammoniacal copper carbonate applied by adding to the water in tubs in which the plants were growing. The Bordeaux mixture is described as giving satisfactory results but as being objectionable on account of its appearance on the foliage. Ammoniacal copper carbonate was preferred to the Bordeaux mixture, but it had to be used in weak solution to prevent injury. The sulphur treatments were unsatisfactory. A secondary advantage from use of the fungicides consisted in the destruction of the green algæ. Spraying with Bordeaux mixture is also recommended by Bisset [2] as an efficient control against the leafspot of pond lilies caused by *Cercospora* sp., but no direct experiment is mentioned.

[1] Halsted, B. D. Experiments with water-lily blight. *In* N. J. Agr. Exp. Sta., 17th Ann. Rpt. [1895]/1896, p. 405–407, fig. 52. 1897.

[2] Bisset, Peter. The Book of Water Gardening . . . p. 188–189. New York, 1907.

In the writer's experiments, carried out in the spring and summer of 1913 at Kenilworth, D. C., Bordeaux mixture (3–3–50 formula) and soda-Bordeaux mixture (2–3–100 formula) were used. In both cases 2 pounds of lead arsenate to 50 gallons of spray mixture were used to combat injurious insects, such as leaf channelers.

The first treatment (May 29, 1913) was applied by spraying the respective mixtures upon the leaves with a hand-pump outfit carried in a boat. Two varieties of *N. odorata* and several varieties of the tender day-blooming blue lilies were treated in this way, with an equal area of unsprayed plants of each variety left as control. Five other similar treatments were given on June 3, 10, 14, 19, and 25. The disease had gained a considerable start before the work was undertaken; hence, the control of the disease was not as complete as it might otherwise have been. However, as shown by careful comparisons of sprayed and unsprayed plots (June 25 to 30), together with leaf counts with respect to injury, the plots sprayed with Bordeaux mixture were clearly 50 per cent less injured by the disease than the control plots. In the plots treated with soda-Bordeaux mixture the control of the disease was almost as high, but in this case a slight spray injury of the leaves was observed.

During the following two seasons this treatment was continued by the owner on a commercial scale with satisfactory results. It might be added that the lead-arsenate treatment was found to be highly efficacious in reducing the injury by leaf channelers.

The ordinary Bordeaux mixture was slightly the better of the two mixtures in controlling the disease, and no spray injury resulted from its use. The soda-Bordeaux mixture, though almost as efficient in its control, and possessed of the advantage of not staining the leaves, caused some spray injury at the strength (2–3–100 formula) and frequency used. Obviously, with frequently submerged foliage, treatments must be made at rather close intervals in order to keep the upper surface of the leaves covered with a film of the fungicide Apparently little or no infection occurred from the lower surface of the leaves.

SUMMARY

About the middle of May, 1913, the attention of the Laboratory of Plant Pathology was drawn to an irregular spotting and decaying of leaves of pond lilies (*Nymphaea* spp.) in the aquatic gardens at Kenilworth, D. C. On account of the severity of the disease in this particular locality and season the present study was undertaken primarily to test the efficacy of spraying the floating leaves of a water plant with ordinary fungicides. However, the causal fungus itself proved so interesting that considerable time has also been devoted to a study of its characters and relation to the host. From the olivaceous-black water-soaked spots

a fungus belonging to the genus Helicosporium has been isolated from leaves collected at Kenilworth, D. C., Arlington, N. J., and New York and Brooklyn, N. Y. The parasitism of this fungus has been demonstrated by successful inoculations made during three years with six different isolations of the fungus on nine species of pond lily (*Nymphaea* spp.). Hitherto, so far as ascertained, no data upon this disease have been published, and the causal fungus is described as a new species under the name *Helicosporium nymphaearum.*

The fungus gains entrance to the host most readily through the stomata, which occur only on the upper leaf surface. Sections of infected leaves show the mycelium ramifying through the intercellular spaces and occasionally between cells which have become separated. Many of the cells of the epidermis and parenchyma become filled with discolored coagulation products and the nuclei and chlorophyll bodies disintegrate. In the older spots the tissues are seen to have more or less completely collapsed and fungus hyphæ are found ramifying both between and within the decaying cells together with bacteria and various protozoa, a condition to be expected in a decaying mass of tissue floating on a watery medium. The sclerotia and multiseptate conidia are developed sparingly on the diseased leaves and rather abundantly on many of the common culture media.

Spraying experiments with ordinary Bordeaux mixture and with soda-Bordeaux mixture were carried out during one season. The disease had gained a considerable start before the work was undertaken; hence, the control of the disease was not as complete as it might otherwise have been. However, as shown by careful comparisons of sprayed and unsprayed plots, the leaves sprayed with Bordeaux mixture were clearly 50 per cent less injured by the disease than were the unsprayed leaves. The control by the soda-Bordeaux mixture was almost as high, but in this case a slight spray injury of the leaves was observed. During the following two seasons this treatment was continued by the owner on a commercial scale with satisfactory results.

PLATE 67

A.—Leaf of *Nymphaea tuberosa* at 1 week after sowing conidia of *Helicosporium nymphaearum* (isolation 205) on upper surface. Note lighter halo around diseased spots. Inoculation of July 7, 1914. ×0.37. Photographed by J. F. Brewer.

B.—Leaf of *Nymphaea* sp. showing spots due to natural infection with *Helicosporium nymphaearum*. ×0.37. Photographed by J. F. Brewer.

C.—Cross section of young sclerotium of *Helicosporium nymphaearum* from a corn-meal-agar culture. ×200. Photomicrographed by J. F. Brewer.

D.—Sclerotium of *H. nymphaearum* from corn-meal-agar culture kept at laboratory temperature for 7 months. Showing germination at 12 hours after sowing in the petri dish of corn-meal agar. ×30. Photomicrographed by J. F. Brewer.

E.—Cross section of old sclerotium developed in a corn-meal-agar slant culture. ×60. Photomicrographed by J. F. Brewer.

A
B
C
D
E

A
B
C

PLATE 68

A, B.—Leaves of *Nymphaea caerulea* at 1 week after sowing conidia of ***Helicosporium nymphaearum*** (isolation 205) on upper surface. Inoculation of July 7, 1914. × 0.75. Photographed by J. F. Brewer.

C.—Leaf of *N. capensis* at 1 week after sowing conidia of *H. nymphaearum* (isolation 225) on upper surface. Inoculation of December 31, 1914. × 0.77. Photographed by J. F. Brewer.

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PLATE 69

A.—Conidium of *Helicosporium nymphaearum* (isolation 205) from culture 3½ months old showing germination in corn-meal-agar petri dish after 12 hours. ×242. Photomicrographed by J. F. Brewer.

B.—Cross section through conidium of *H. nymphaearum* showing thin spot or perforation in cross wall and surface sculpturings on outer wall. ×900. Photomicrographed by J. F. Brewer.

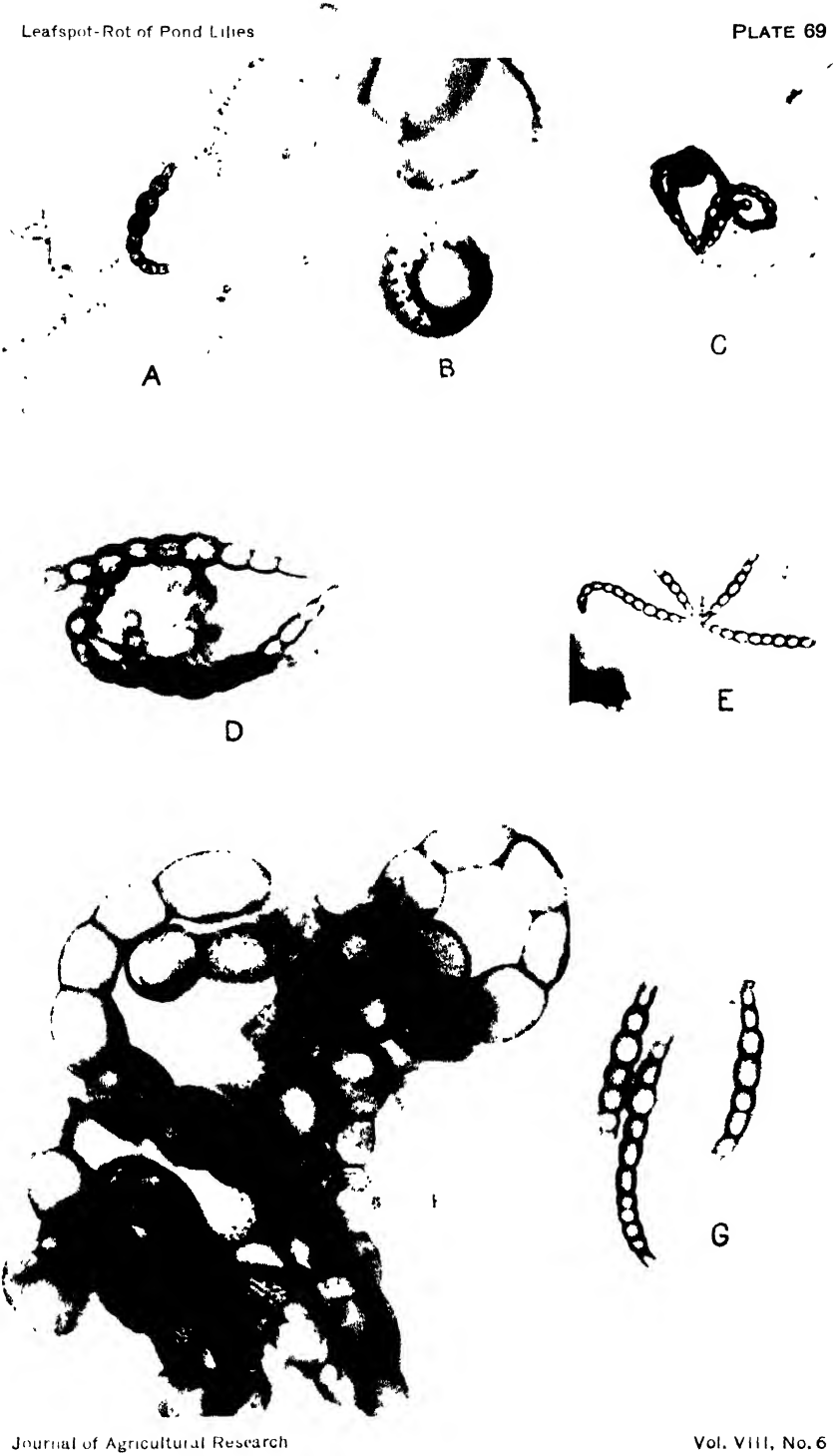
C.—Conidial group (*H. nymphaearum*) *in situ* in petri-dish culture of corn-meal agar at 2 weeks. ×107. Photomicrographed by J. F. Brewer.

D.—Conidia of *H. nymphaearum* showing attachment to sporophore and sculpturings on the outer walls. ×285. Photomicrographed by J. F. Brewer.

E.—Conidial group (*H. nymphaearum*) in corn-meal-agar petri-dish culture at 13 days. ×107. Photomicrographed by J. F. Brewer.

F.—Water mount of conidia of *H. nymphaearum* from a corn-meal-agar petri-dish culture 13 days old, showing echinulate sculpturings on outer spore walls. ×828. Photomicrographed with oil-immersion lens by Dr. Erwin F. Smith.

G.—Conidia of *H. nymphaearum* from corn-meal-agar culture showing sculpturings on outer spore walls. ×285. Photomicrographed by J. F. Brewer.


A
B
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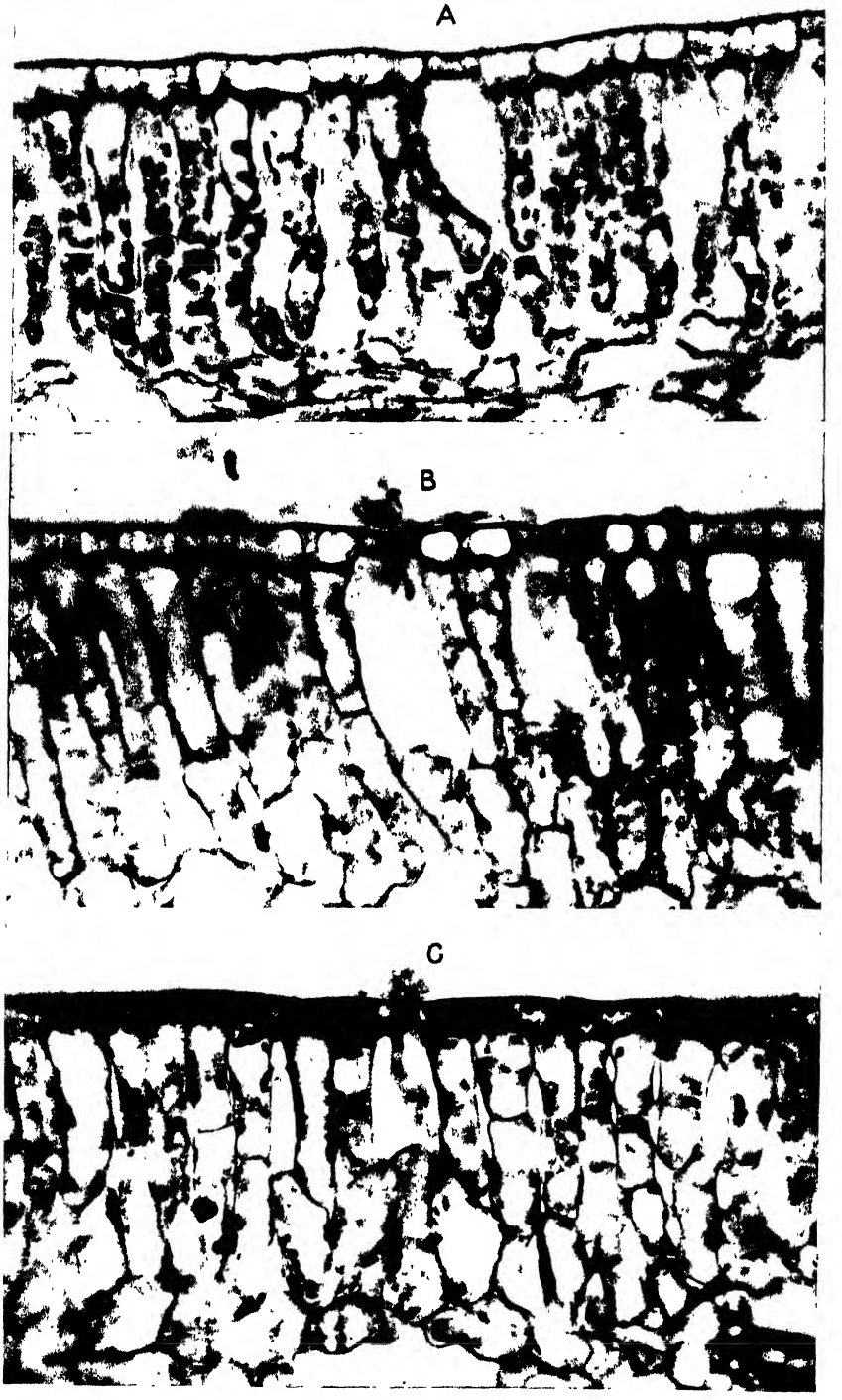
A
B
C

PLATE 70

A.—Cross section of normal part of leaf blade of *Nymphaea odorata* the diseased part of which is shown in B. ×430. Photomicrographed by J. F. Brewer.

B.—Cross section of leaf of *N. odorata* at four days after sowing of conidia of *Helicosporium nymphaearum* from pure culture to upper surface of leaf. Note penetration of stomatal opening by germ tube of the fungus. ×430. Photomicrographed by J. F. Brewer.

C.—Cross section of leaf of *N. odorata* at four days after sowing of conidia of *H. nymphaearum* from pure culture to upper surface of leaf. Showing penetration of fungus through stomatal opening and further growth of hyphæ in substomatal chamber. ×430. Photomicrographed by J. F. Brewer.

Compare figures A to C for progressive disintegration of cellular contents of host after entry of the fungus, coagulation of contents of epidermal cells and disintegration of nuclei and chlorophyll bodies.

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STUDIES OF THE GENUS PHYTOPHTHORA

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INTRODUCTION

From a study of one of the species of Phytophthora it appeared that there was a great need of a comparative study under the same conditions of all the described species of the genus. Hitherto, the various species had never been gathered and grown by any one investigator; nor had comparisons been made when grown under similar conditions; nor had any general survey of the genus been made since it was established, although species had been added from time to time. The cytology of the genus has likewise been almost entirely neglected. As a result of this, together with the imperfections in the descriptions in some cases, it is almost impossible to identify a species with any degree of accuracy. Moreover, because of the scarcity of spore forms on some of the original hosts, as well as their variation when present, herbarium specimens are practically useless for purposes of identification. Another reason which has doubtless tended to check work with this genus is the difficulty encountered in growing some of the species of Phytophthora in culture, even after they have been obtained pure on artificial substrata.

In this group of fungi it is often not sufficient in making transfers to remove a bit of mycelium. The cœnocytic nature of the mycelium necessitates the transfer of an especially long strand so that the cells may remain unbroken. They are so difficult to grow that some of the species, such as *P. thalictri* and *P. agaves*, the latter of which has not yet been described, have not so far been reported in culture.

The object of this paper is to present the results of the writer's studies of the genus from a systematic and a biometrical standpoint. A tenta-

[1] The writer began this work in the Department of Plant Pathology of Cornell University, and completed the major portion of it as a member of the Office of Cotton, Truck, and Forage Crop Disease Investigations. He wishes gratefully to acknowledge the many courtesies shown him by Prof. H. H. Whetzel and Dr. Donald Reddick, in whose laboratories the work at Cornell University was carried on; the valuable suggestions received from Dr. Raymond Pearl, of the Maine Agricultural Experiment Station, and Dr. H. H. Love, of Cornell University, in connection with the biometrical portion of the paper; and the aid given by Mrs. N. E. Fealy, of the Department of Agriculture, in editing the manuscript.

tive table is offered for the separation of species in the hope that it will aid other investigators in identifying the forms with which they work. This paper should be followed by others on parasitism, host relationships, and cytology of all forms that will throw light on the systematic relationship of the species. When these studies are completed, it will perhaps be possible to arrange a more natural classification.

ECONOMIC IMPORTANCE OF THE GENUS PHYTOPHTHORA

Although the actual number of species of Phytophthora is small, geographically they are very widespread, their presence having been recorded from the Tropics as well as the temperate regions, including Japan, India, Java, most of the countries of Europe, Australia, the East Indies, North America, and South America. Botanically, the hosts of *Phytophthora* spp. are distributed among 15 families, ranging from the Pinaceae to the Scrophulariaceae (16)[1], and including plants of all ages and textures. On account of its omnivorous character, the injuries it causes are serious. These injuries include the damping-off of seedlings, spots on leaves, the rotting of fleshy tubers and rhizomes, and cankers on woody stems. The genus has attracted most attention through the attacks of one of the species on the common potato (*Solanum tuberosum*). Although a number of our leading pathologists have devoted their best efforts to the study of this species, considerable work on its life history and cytology still remains to be done.

The genus Phytophthora was founded by De Bary (1) on the potato-blight fungus. Up to the present time Saccardo has enumerated the following species of the genus:

Phytophthora infestans (Mont.) De Bary (1).
Phytophthora cactorum (Cohn and Lebert) Schroeter (31).
[*Peronospora cactorum* Cohn and Lebert (18).
Peronospora fagi Hartig (14, 15), *Peronospora sempervii* Schenk (30), *Peronospora omnivora* De Bary (2)]
Phytophthora phaseoli Thaxter (34).
Phytophthora colocasiae Raciborski (25).

The following species should now be added:

Phytophthora nicotianae Van Breda de Haan (3).
Phytophthora thalictri Wilson and Davis (36).
Phytophthora syringae Klebahn (17).
Phytophthora faberi Maublanc (19).
Phytophthora arecae (Colem.) Pethybridge (23), (24).
Phytophthora parasitica Dastur (10).
Phytophthora erythroseptica Pethybridge (24).

Another species, *P. agaves* Gandara, has been mentioned in literature (13); but as yet no description has been given, and no cultures have been distributed.

[1] Reference is made by number to "Literature cited," pp. 273–276.

As the contributions to the genus in recent years are well reviewed in a paper by Guy West Wilson (37), the writer will not attempt to review them here.

EXPERIMENTAL METHODS EMPLOYED

Pure cultures were obtained from as many of the foregoing species and from as many sources as possible. Of the 11 species[1] described, nine were studied and grown in pure culture. In addition to these, cultures of *P. fagi*, which is given in Saccardo as a synonym of *P. cactorum*, were also obtained. It was not possible to procure fresh material of *P. thalictri* or *P. colocasiae*. The following is a list of the cultures used and sources from which they were obtained:

P. infestans, isolated from potatoes from New York and Maine by the writer, and received from G. P. Clinton, Connecticut, I. E. Melhus, Wisconsin, and S. Ito, Japan.

P. cactorum, isolated by the writer from ginseng from Ohio, Michigan, New York, and Pennsylvania, and by D. L. Peters from a species of Phyllocactus in Germany.

P. phaseoli, isolated by the writer from bean pods from New York, and received from G. P. Clinton, Connecticut.

P. nicotianae, isolated from tobacco in Germany, received from H. H. Whetzel, and from tobacco in Japan, received from S. Ito.

P. syringae, isolated from lilac in Germany, received from H. H. Whetzel, and from lilac in Ireland, received from George H. Pethybridge.

P. arecae, isolated from areca palm in India, received from L. C. Coleman.

P. parasitica, isolated from castor bean, received from J. F. Dastur, India, and G. W. Wilson, New Jersey, the latter culture having probably also come from Dastur.

P. faberi, isolated from cacao in Trinidad, received from J. B. Rorer.

P. jatrophae, isolated by Jensen in Java from one of the nettles and obtained from the Centralstelle für Pilzkulturen, Amsterdam, Holland.

P. fagi, isolated by George H. Pethybridge from young plants of *Fagus* spp. in Ireland, and obtained from Centralstelle für Pilzkulturen, Amsterdam, Holland.

The study of the cultures was supplemented with a study of herbarium material of most of the species.

The ideal method of procedure would have been to study these forms on the original host in each particular case, as in that way no variation in size or form could be attributed to an unnatural substrata relationship; but when the work was begun this was impossible, because the various hosts were not available. The aim was, however, to subject all the forms to identical conditions; and with this in view, a number of artificial media were tried in a preliminary way in order to determine which was most favorable for all the forms.

It was found that the various forms reacted differently on the different media both as regards rate of growth and spore forms produced. The greater number of species made a good growth and produced an abundance of spore forms on oat agar made, with slight modifications, accord-

[1] Prof. S. Ito recently informed the writer that Mr. K. Sawada, of the Formosa Agricultural Experiment Station, lately described in Japanese two new species of Phytophthora occurring on *Allium fistulosum* and *Solanum melogena*, respectively.

ing to the directions given by G. P. Clinton (7). Unless otherwise stated, therefore, this was the media used in all cases where measurements and drawings were made.

In order to obtain a definite spore form, it was necessary in some cases to employ special substrata, such as cooked carrot, potato cylinders, corn-meal agar, and sterilized flies. As artificial media were most generally employed, it seemed important to determine whether these influenced the morphology and size of the various structures to any appreciable extent. Preliminary trials were made with *P. infestans* on potato foliage, but no appreciable differences between the spores produced on this and on those grown on artificial media could be detected. The morphology and size of the various structures of a number of other forms as they occur on the original host are now being studied, and in a later paper these studies will be compared with those recorded here.

The observations and measurements were made at the time of the first appearance of conidia and oospores, at which stage the culture most nearly approaches normal. Soon after this, if the cultures are kept at growing temperatures, the spore forms begin to show various abnormalities, chief among which may be mentioned various proliferations and the formation of secondary conidia.

The temperature at which cultures are grown is a factor in the production of normal and comparable cultures, low temperatures tending to diminish the size of the spore forms and the higher temperatures tending to diminish their production. The cultures from which these studies were made were kept at room temperature varying from 18° to 20° C. No records were kept of the number of times they were transferred. In making the transfers it was found that, if the substrata and temperature conditions were the same, the spore forms appearing were also the same, no matter whether mycelia, conidia, or sexual bodies were used in the transfer.

The purity of the cultures was tested as often as seemed necessary. Several methods were employed in the tests. If the presence of bacteria was suspected or if the sole object was to determine the presence of bacteria, transfers were made from the cultures to nutrient beef agar, a medium on which species of Phytophthora make no growth, but which is especially favorable to the growth of bacteria. In the case of forms which produced an abundance of conidia, dilution plates were made and the cultures recovered from colonies resulting from single conidia. This was a long and tedious process, however, as generally only a small percentage of the conidia germinate on the poured plates. More recently a modification of the above method was used. Where there was a doubt as to purity, the culture was transferred to an Erlenmeyer flask containing media known to produce an abundance of conidia. When the conidia appeared and were in a normal condition, sterilized water was

poured into the flask under aseptic conditions and the culture placed at a temperature favorable for the germination of the conidia by means of swarm spores (20). When such germination took place, each conidium gave rise to from 20 to 40 swarm spores. Poured plates were then made from this solution which contained hundreds of zoospores the majority of which germinated readily on the agar. Instead of obtaining a culture from a single conidium, which is potentially a multiple of zoospores, the new culture was obtained from a single zoospore.

After making a number of measurements of conidia and observing the great variation in size, it occurred to the writer that by continual platings from large selected individuals cultures could be finally obtained which would produce only large conidia, and vice versa. To determine this point, dilution plates were made, a number of single large conidia were marked, and the colonies resulting from one or more of these transferred to agar slants. When these cultures reached the normal condition in the production of conidia, counts were made to determine the percentage of large and small conidia. Dilution plates were again made and the large conidia marked, and the colonies resulting from this second growth or generation were again transferred to agar slants. When the normal spore-producing stage was reached, the percentage of large and small conidia was again determined. This procedure was repeated through five generations, the large conidia being selected in all cases. There was no material difference between the percentage of large and small conidia in the original culture and the culture resulting from the last selection. This indicated that, notwithstanding the great variation in size, the limits and percentages of large and small conidia remain practically constant and are therefore good characters to be employed in taxonomy.

The characters to be relied on in taxonomic work must be constant under all conditions and not correlated with certain adaptations to habitat. While there are advantages in employing characters which show relationship between the species, this is not always possible. In the work here set forth the separation and the relationship of species are made on the aggregate of characters, it being borne in mind that the proportionate value to be attached to each character must necessarily vary.

The most valuable characters that can be employed are doubtless those which are exhibited by the reproductive organs and which, as will be seen, furnish means of separating the genus into different groups. Other characters which may be conveniently used in separating the various species, arranged as nearly as possible in the order of their importance, are (1) the size and morphology of the sexual reproductive bodies, conidia, chlamydospores, and other structures; (2) shape of the terminal papillum in the conidium; (3) the ratio of the length to the width in the conidia; (4) variation in the mycelium; and (5) the microscopic growth, time of appearance of spores, and kind of spore forms produced on various media.

MACROSCOPIC GROWTH ON VARIOUS MEDIA

The media used in making these comparisons were Thaxter's hard potato agar, oat agar, Lima-bean agar, string-bean pods, and corn-meal agar.[1] Various synthetic media were also used; but owing to the small amount of growth produced, they were unsuitable for this work.

Five different series, consisting of three cultures each of the various forms, were made in test tubes and grown at room temperatures. Owing to differences in the hardness of the media, amount of moisture, air, etc., there were slight differences in the amount of aerial mycelium in the different series; but in general the results in all were practically identical and uniform.

As a minor character, the appearance of the macroscopic growth on a given media is of some value when taken with other distinguishing characters. Plantings of the different forms on potato agar poured in large petri dishes, which offer more uniform conditions than can be obtained in test tubes, also showed differences in the luxuriance of growth in some of the species. The distinguishing characters, however, are best shown when grown in test-tube slants. Potato agar was found to be best suited for this purpose. A summary of results of the growth on this medium 12 to 18 days after the transfers were made showed that the cultures may be divided into three groups, as follows: (1) Growth very profuse, aerial, and fluffy. In this group are included *P. parasitica*, *P. fagi*, *P. jatrophae*, *P. cactorum*, and *P. faberi*. (2) Growth less profuse, more or less embedded in substratum, lacking the fluffy appearance, irregular surface, often forming numerous pellicular-like growths and presenting a granular appearance. In this group are included *P. syringae* and *P. nicotianae*. (3) Very little or no growth. In this group are included *P. infestans* and *P. phaseoli*.

On oat agar, unlike the potato agar, *P. syringae* alone could be distinguished at all times from the remaining forms. The character of growth for this form was similar to that on potato agar. All the other forms make a profuse white fluffy growth on the oat agar.

On bean pods no difference could be detected, all forms making a good, fluffy growth except *P. infestans* and *P. phaseoli*, which made little or no growth.

On corn-meal agar no difference could be detected between the different forms, but all made scantier growth than on the other media.

On Lima-bean agar all forms, including *P. infestans* and *P. phaseoli*, made a profuse white growth.

[1] Thaxter's hard potato agar, 200 gm. of potato, 20 gm. glucose, and 15 gm. of agar for every 1,000 c. c. of water; Clinton's oat agar, made according to the directions given by Clinton (8), except that rolled oats and 7½ gm. of agar for every 1,000 c. c. of media were used; bean-pod plugs, consisting of pods of ordinary string beans placed in test tubes with a small quantity of water and sterilized; corn-meal agar, made according to the directions given by Shear and Wood (32); Lima-bean-juice agar, made according to the directions given by Dastur (10).

KIND OF SPORE FORMS PRODUCED AND TIME OF APPEARANCE

Microscopic examinations from time to time of cultures made in test tubes on hard potato agar, oat agar, Lima-bean agar, corn-meal agar, and bean pods, and grown at room temperatures, showed that even when grown under as nearly identical conditions as possible the different cultures did not produce the same spore forms.

As will be seen in Table I, which shows the spore forms found at the end of two and six weeks, certain of the media are more favorable for the production of all the spore forms than others, and oat agar is the most suitable, especially as regards the sexual bodies. It should be borne in mind that the production of the spore forms on the various media shown in the table is not absolute, but what may be expected under normal conditions. This table was compiled as the result of observations during the past three years.

TABLE I.—*Spore forms produced by species of Phytophthora on various media at the end of two and six weeks*

TWO WEEKS

Culture.	Potato agar.	Oat agar.	Lima-bean agar.	Bean pods.	Corn-meal agar.
P. jatrophae	No spores	Conidia; chlamydospores	Conidia; chlamydospores (few).	Chlamydospores (few)	Conidia (few).
P. parasitica	do	Conidia (few)	Conidia	No spores	Conidia (few); chlamydospores.
P. arecae	Conidia (few)	Oospores (few)	Conidia· oospores (few)	No spores	No spores.
P. nicotianae	No spores	No spores	No spores	Chlamydospores (few)	Chlamydospores (few).
P. fagi	Conidia	Conidia; oospores	Conidia; oospores	Conidia· oospores	Conidia (few); oospores.
P. infestans	No spores	Conidia	Conidia	Conidia (few)	Conidia (few).
P. faberi	Chlamydospores	Conidia; chlamydospores	do	Conidia; chlamydospores	Conidia; chlamydospores (?).
P. cactorum from *Panax* sp	Conidia	Conidia; oospores	Conidia; oospores	Conidia; oospores (few)	Conidia (few); oospores.
P. cactorum from *Phyllocactus* sp	do	do	Conidia; oospores (few)	Conidia; oospores	Conidia; oospores.
P. erythroseptica	No spores	Oospores	No spores	Oospores	Oospores.
P. phaseoli	do	Conidia; oospores	Conidia	Conidia	Conidia; oospores (few).
P. syringae	do	No spores	No spores	No spores	No spores.

SIX WEEKS

P. jatrophae	Chlamydospores (few)	Conidia (few); chlamydospores.	Conidia (few); chlamydospores (few).	Conidia; chlamydospores (few).	Chlamydospores.
P. parasitica	No spores	Conidia (few)	Conidia	No spores	Conidia; chlamydospores.
P. arecae	Conidia (few)	Conidia; oospores [a]	Conidia (few); oospores (few).	Conidia (few); oospores (few).	Conidia.
P. nicotianae	Chlamydospores (few)	Chlamydospores (few)	Conidia; chlamydospores (few).	Conidia; chlamydospores	No spores.
P. fagi	Conidia	Conidia (few); oospores	Conidia; oospores	Conidia (few); oospores	Conidia; oospores.
P. infestans	No spores	Conidia; oospore-like bodies [b]	Conidia (few)	Conidia	Conidia.
P. faberi	Conidia; chlamydospores	Conidia (few); chlamydospores.	Conidia; chlamydospores	Conidia; chlamydospores	Conidia; chlamydospores.
P. cactorum from *Panax* sp	Conidia	Conidia; oospores	Conidia; oospores	Conidia; oospores	Conidia (few); oospores.
P. cactorum from *Phyllocactus* sp	Conidia; oospores (few)	do	do	do	Do
P. erythroseptica	No spores	Oospores	No spores	Oospores	Oospores.
P. phaseoli	do	Conidia; oospores	Conidia (few)	Conidia	Conidia (few).
P. syringae	do	Conidia (few)	No spores	Oospores	Conidia.

[a] Numerous oospores were produced on oat agar soon after the culture was received in 1913. Recent examination of transfers from the original culture show very few or no oospores.

[b] See text for discussion of these bodies.

In general the time of appearance of the spore forms from different strains of the same species on a given medium may vary, but eventually the same form appears. The kind of spore forms and the time of their appearance, as in the case of the macroscopic appearance of the growth, may be influenced by the length of time the fungus is kept in culture; and consequently these characters can be of little value in a systematic separation of the species, except when used in conjunction with more stable differences. The production and appearance of certain spore forms in some of the cultures of *P. infestans* will be discussed in a later paragraph.

COMPARATIVE MORPHOLOGY

MYCELIUM

The mycelium of all the species is white and aerial or submerged, as shown by the growth on the artificial substrata previously mentioned, and the hyphæ when young are cœnocytic and filled with a dense, granular protoplasm, oftentimes intermingled with larger particles, probably fat and glycogen. In old cultures, where the supply of nutrition is not so abundant, septation may often be seen; and at this stage the granular protoplasm becomes less dense, and many vacuoles appear. Since the tips of the hyphæ are the young growing parts it would appear that the granules are translocated thither from the older basal portions of the hyphæ.

The appearance of the hyphæ varies, depending on whether the mount was made from aerial or from submerged growth, the aerial mycelium being straight and the submerged much twisted and gnarled.

The mycelia of the different species do not show any distinguishing characters when grown on oat agar. Plate 71, *B*, shows the mycelium of *P. syringae* on this agar, and this is the general appearance of the mycelium of the other forms when grown on this medium.

On potato agar the character of the mycelia is similar to that on oat agar, except in the case of *P. syringae*, in which species the hyphæ present a gnarled and tuberculate appearance (Pl. 71, *A*). This character makes the mycelium of *P. syringae* readily distinguishable from the other forms when grown on potato agar. Ward (35) observed a similar appearance in the mycelium of *Pythium gracile*, and suggested that these structures may serve as reservoirs of protoplasm for the immediate use of the mycelium or for the formation of oogonia. Butler (4) likewise observed structures in the same genus and compared them to the toruloid structures often formed by fungi, the function of which is to carry on a vegetative propagation of the species. Butler's drawings, in his Plate 1, figures 3 and 4, bear a striking resemblance to the figure shown by the writer, to whom these structures appear to be more nearly homologous with sclerotia, though lacking the texture of the latter. The tuberculate protuberances are slightly darker, densely packed with protoplasm, and much more capable

of withstanding adverse conditions than the ordinary hyphæ. These protuberances on the mycelium of *P. syringae* grown on potato agar can be used to advantage in separating it from the other species. It is practically impossible to distinguish any of the other species by their mycelial characters with any degree of accuracy. It should be noted, however, that the mycelium of *P. nicotianae*, whether grown on oat or potato agar, generally contains larger and a greater number of the globoid particles of a fatty or glycogen nature than any of the other species.

The size of the mycelia varies greatly, as shown by measurements made from aerial and from submerged growth. The following are the limits of variation for each of the different forms:

FORM.	SIZE. μ
P. parasitica	1.91 to 7.66
P. infestans	2.87 to 11.49
P. phaseoli	2.87 to 11.49
P. syringae	2.87 to 7.66
P. nicotianae	2.87 to 17.23
P. jatrophae	1.27 to 7.66
P. arecae	1.91 to 7.66
P. cactorum	1.91 to 7.66
P. faberi	1.91 to 7.66
P. fagi	1.91 to 7.66
P. erythroseptica	1.91 to 7.66

CONIDIOPHORES

As several of the forms produced very few or no conidia-bearing hyphæ on the ordinary agars previously employed, it was necessary to grow some of them on special media in order to study the conidiophores. For example, in the case of *P. erythroseptica*, it was necessary to use flies as the medium in order to obtain conidiophores. Owing to the great length of these structures and the manner in which spores are borne, they can not be studied under high magnification.

The general procedure followed varied according to the form. By means of the following method the majority were easily studied: Van Tieghem cells were carefully cleansed and placed in a large moist chamber and sterilized. Clean cover glasses were flamed, and a drop or two of melted media placed on each and allowed to harden, at the same time all possible precautions being taken to prevent contamination. After this each drop was inoculated with a pure culture of the form to be studied, and the cover glasses then inverted on the Van Tieghem cells, at the bottom of which a few drops of sterile water had previously been placed. The cover glasses were held in place by means of sterilized vaseline. At the end of not less than 36 hours the conidiophores and conidia began to appear and were easily examined with the low magnification of the microscope.

The number of conidia found on a single conidiophore varies from a minimum of 2 to a maximum of more than 20. The sympodial type of branching prevails. Plate 72 shows representative types of conidiophores in the various forms made with the 7.5 ocular and 16 mm. objective. As shown in this plate, such forms as *P. phaseoli* and *P. nicotianae* are more prolific in the production of conidia than others. Examination of the conidiophores of *P. infestans*, *P. phaseoli*, and *P. thalictri* with a higher magnification showed that they differ from the other forms in that there is a slight thickening of the conidiophore immediately below the point of attachment. This character is constant on both the natural substratum and in the artificial cultures, as shown by both *P. infestans* and *P. phaseoli*. The conidiophores vary greatly in size, the variation ranging from 200 to 500 μ in length and 3.5 to 8.5 μ in width.

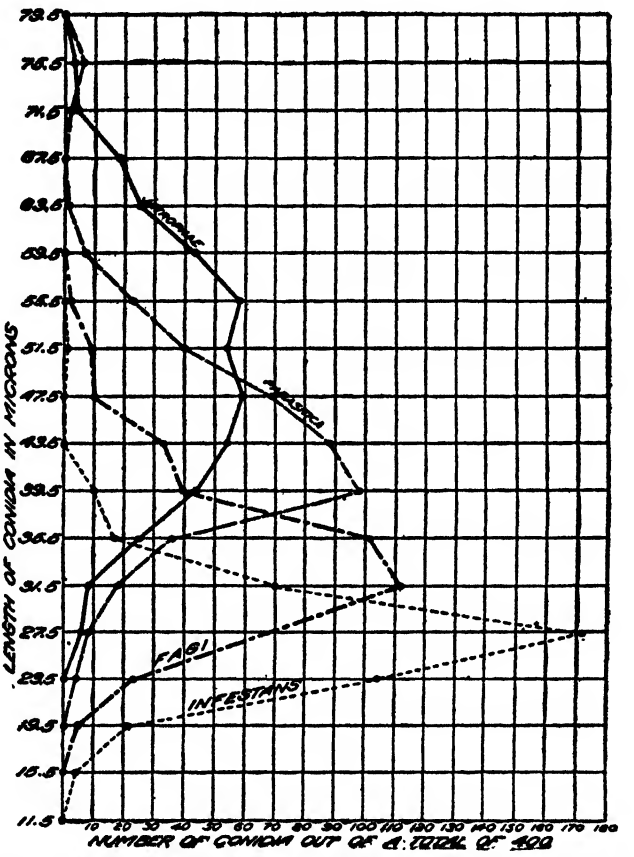

FIG. 1.—Graph showing the variation in length of conidia of *Phytophthora infestans*, *P. fagi*, *P. parasitica*, and *P. jatrophae*.

CONIDIA

Conidia are formed in all the species of Phytophthora studied, and are almost always terminal, although in rare instances structures are seen which may be intercalary conidia. They are elliptical to ovate, approaching the globose, with a more or less prominent apical papillum. In all the forms the color of the conidia is the same—that is, pale gray to colorless. The contents in all cases are finely granular, sometimes with a large central vacuole. The size of the granules and the presence of vacuoles depend on the age of the conidium. As it advances in age the protoplasm and granules appear to divide and become less dense and the number of vacuoles may also increase. Neither the color nor

the contents of the conidia show any distinguishing characters (Pl. 73, 74).

MEASUREMENTS OF CONIDIA.—Measuring spore forms to delineate species in the various fungus groups is a well-established practice. In forms less variable than those of *Phytophthora* spp. a small number of such measurements might be sufficient to determine the average size. The conidia of species of Phytophthora, however, are so variable that a small number of measurements are likely to be misleading. This is brought out by the following figures, which show the variation in the measurements of the same species as given by 12 different workers.

Name of worker.	Size of conidia.	Size of oospores.
	μ	μ
Cohn and Lebert	45 by 35 to 68	20 by 70.
Hartig	25 to 40 by 25 to 40	
Schenk [1]		20 (oogonium).
De Bary	35 to 40 by 50 to 60 to 93	16 to 24.[2]
Schroeter	35 to 40 by 50 to 60	24 to 30.
Osterwalder	14.64 to 24.4 by 119.56	24 (oogonium).
Himmelbauer	Not given	30 to 45.
Zimmerman	17 to 30 by 25 to 60	None found.
Hori	30 to 50 by 50 to 60	26 to 28.
	Abnormal, 29 by 85.5	
Bubak	15 to 25 by 15 to 120	Not given.
Van Hook	30 to 42 by 40 to 58	Do.
Writer	15 to 30 by 18 to 54	21 to 36.

[1] "Die kleinsten derselben sind 5, die grossten 36 Theilstriche meines zeissechen Mikrometers lang und 4 bis 25 Theilstriche breit."

[2] De Bary states that the oospore is in general three-fourths to four-fifths the diameter of the oogonia, and gives 24 to 30 μ as the diameter of the latter, so that the diameter of the oospore given above was obtained accordingly.

As the measurements given above, if correct, would warrant the establishment of more than one species, it seemed advisable to make a large number of measurements of all the forms in culture and present them in their entirety. Accordingly measurements of conidia were made from normal oat-agar cultures of relatively the same age—that is, cultures which had just begun to form conidia, except in the case of four species: (1) *P. erythroseptica*, which was obtained from normal cultures grown on sterilized flies; (2) *P. parasitica*, and (3) *P. nicotianae*, obtained from normal cultures grown on Lima-bean agar, and (4) *P. syringae* from sterilized carrots or bean pods. The scarcity of conidia of these four species on oat agar made the use of special media necessary. It is realized that error may have resulted from the use of different substrata for growing the various forms; but in preliminary trials with a number of different artificial substrata the size of the spore forms remained practically constant on all.

The measurements, of which there were two sets of 200 conidia each, are presented in Table II. These measurements were made at different times and show the variation that may reasonably be expected. The

table gives the class in microns, the number of conidia out of a total of 200, both for length and width, falling into each class, and the results of combining the data of sets A and B. In figures 1 to 6 curves are plotted for the length and width of conidia in microns, and the number of conidia falling into each class out of a total of 400 measurements. These curves were obtained by combining the data given in Table II and making the classes differ by 4 μ instead of 2 μ. They show at a glance the variation that may reasonably be expected in each species,

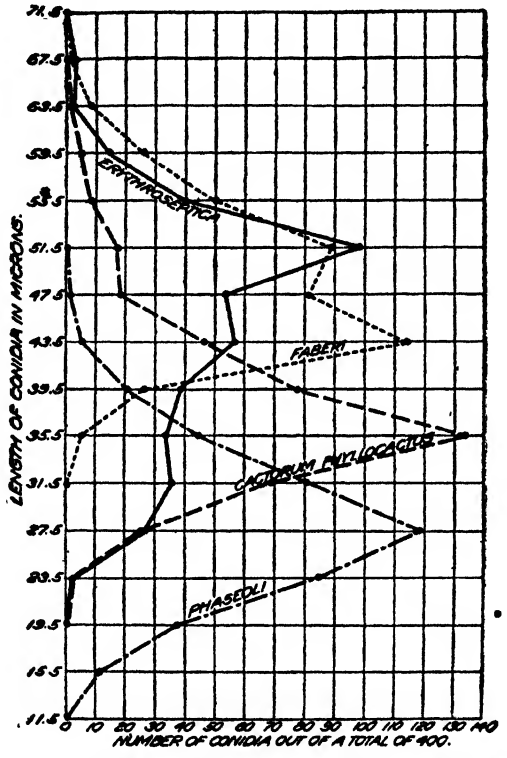

FIG. 2.—Graph showing the variation in length of conidia of *Phytophthora phaseoli*, *P. cactorum* (*Phyllocactus* sp.), *P. faberi*, and *P. erythroseptica*.

as well as the class containing the greatest number of individuals for each species. Further examination of these curves shows that some of them, as, for example, the length of *P. jatrophae*, exhibits a mode of 47 μ and of 55.5 μ. This is probably not to be interpreted as meaning that the normal-sized variation curve is a multimodal one, as too few observations or too numerous classes may cause a similar condition. A similar condition is indicated by the length of *P. nicotianae*. Theoretically all these curves are smooth, and probably would be if an infinite number of measurements were made.

TABLE II.—*Summary of measurements of two sets of conidia of species of Phytophthora*

SET A

Class.	*P. cactorum* (*Panax* sp).		*P. fagi.*		*P. faberi.*		*P. jatrophae.*		*P. nicotianae.*		*P. arecae.*		*P. parasitica.*		*P. infestans.*		*P. phaseoli.*		*P. syringae.*		*P. cactorum* (*Phyllocactus* sp).		*P. erythroseptica.*	
	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.
μ																								
9.5 to 11.49																4		2						
11.5 to 13.49														1		9		2						
13.5 to 15.49														7		5	1	17		2				
15.5 to 17.49		1		4								1		8	4	46	2	29		1				
17.5 to 19.49		3	2	12		1		6				2		23	6	59	10	51		14				
19.5 to 21.49		11	3	19		1		1		2		0		30	10	38	6	37		19				
21.5 to 23.49	2	50	3	28		6		2		10		8	2	44	28	19	16	42	3	24				
23.5 to 25.49	1	30	4	27		4		4	1	6		14	2	22	28	4	17	12	5	24				
25.5 to 27.49	10	52	11	37		16	3	20	1	16	2	31	4	17	51	14	28	7	9	50				
27.5 to 29.49	4	23	14	18		28	0	12	2	16	1	20	3	10	35	1	23	0	4	28				
29.5 to 31.49	20	24	26	25		38	2	22	31	32	7	38	3	15	21	1	35	1	15	22				
31.5 to 33.49	8	3	25	12		12	3	21	7	20	3	23	6	7	10		16		3	5				
33.5 to 35.49	50	2	38	9	4	37	10	38	19	36	12	30	0	9	7		16		15	8				
35.5 to 37.49	3	1	10	5	1	20	9	14	4	10	5	6	8	2			8		8	0				
37.5 to 39.49	44		22	2	5	18	25	28	29	20	37	13	27	4			13		19	1				
39.5 to 41.49	11		2	0	1	13	8	9	16	9	7	5	10	0			3		5	0				
41.5 to 43.49	22		16	2	26	6	25	9	21	10	20	5	20	1			4		23	1				
43.5 to 45.49	13		7		35	0	4	5	8	9	9	3	12				1		9	0				
45.5 to 47.49	2		5		9		21	2	28	3	35	1	33						21	0				
47.5 to 49.49	4		3		20		12	2	10	1	13		9						10	0				
49.5 to 51.49	5		6		40		18	4	3		3		19						12	0				
51.5 to 53.49	0		2		20		12	1	9		3		10						5	0				
53.5 to 55.49	0		1		23		20		6		11		17						10	0				
55.5 to 57.49	0				1		5		2		0		3						2	1				
57.5 to 59.49	0				9		11		2		4		4						2					
59.5 to 61.49	0				2		4		0		12		0						2					
61.5 to 63.49	1				3		4		0		5		0						5					
63.5 to 65.49					1		0		1		0		0						1					
65.5 to 67.49							2				1		0						0					
67.5 to 69.49							1				0		0						1					
69.5 to 71.49							1				4		2						2					
71.5 to 73.49											1		0						0					
73.5 to 75.49											1		6						2					
75.5 to 77.49											0								0					
77.5 to 79.49											1								1					
79.5 to 81.49											0								0					
81.5 to 83.49											0								2					

83.5 to 85.49												0								1						
85.5 to 87.49												1								2						
87.5 to 89.49												2								1						

SET B

9.5 to 11.49											1						1		3							
11.5 to 13.49											1						4		5							
13.5 to 15.49										1	2		7		1		30	2	20		2					
15.5 to 17.49					5					0	16		0		16	3	68	11	68		19					
17.5 to 19.49		8		21						3	17		4		52	2	26	10	34	1	25					
19.5 to 21.49		25	4	50		1		1		9	35		16		56	29	3	30	20	4	26					
21.5 to 23.49	1	34	12	35		4		2		8	22		17		53	19	0	21	6	9	37					
23.5 to 25.49	2	77	19	55		28		9	19	30			48		15	60	2	47	3	17	42					
25.5 to 27.49	6	24	23	15		35	3	5	15	17			18	1	6	27		20		5	23					
27.5 to 29.49	18	23	41	13		42	3	17	20	21	1		41	3	0	29		21		20	18					
29.5 to 31.49	19	4	20	2		20	0	9	14	14	2		12	6	1	10		8		15	3					
31.5 to 33.49	61	2	37	2		28	3	14	29	8	3		11	13		8		12		28	2					
33.5 to 35.49	19	2	16	1		12	3	10	14	5	4		10	15	0	2		8		17	1					
35.5 to 37.49	44	1	11	1	9	20	7	23	20	4	8		8	39		6		3		25						
37.5 to 39.49	6		4		11	4	3	12	10	6	6		2	22		4		1		15						
39.5 to 41.49	11		9		30	4	19	19	12	1	16		5	32						15						
41.5 to 43.49	2		1		23	1	6	12	6		11			24						9						
43.5 to 45.49	6		1		35	0	19	21	10		34			12						12						
45.5 to 47.49	3		1		17	1	7	9	4		18		1	15						4						
47.5 to 49.49	1		1		20		17	19	4		27			7		1				3						
49.5 to 51.49					9		7	3	1		16			4						1						
51.5 to 53.49					15		24	5	1		16			1												
53.5 to 55.49					11		9	5			8			2												
55.5 to 57.49	1				10		19	4			9			1												
57.5 to 59.49					5		9	1			2			2												
59.5 to 61.49					3		15				6			1												
61.5 to 63.49					1		6				4															
63.5 to 65.49					1		11				3															
65.5 to 67.49							4				3															
67.5 to 69.49							2				2															
69.5 to 71.49							1				1															
71.5 to 73.49							2																			
73.5 to 75.49							1																			
75.5 to 77.49																										

SUMMARY OF SETS A AND B

9.5 to 11.49											1						5		5							
11.5 to 13.49											1			1			13		7							
13.5 to 15.49										1	2		8	9	4		112	8	70		3					7
15.5 to 17.49		1		4					1	2			8	9	4		112	8	70		3					7
17.5 to 19.49		3	2	27		1		6	0	16			2	39	9		127	21	119		33		9			24

TABLE II.—*Summary of measurements of two sets of conidia of species of Phytophthora*—Continued

SUMMARY OF SETS A AND B—continued

Class.	*P. cactorum* (*Penax* sp).		*P. fagi.*		*P. faberi.*		*P. jatrophae.*		*P. nicotianae.*		*P. arecae.*		*P. parasitica.*		*P. infestans.*		*P. phaseoli.*		*P. syringae.*		*P. cactorum* (*Phyllocactus* sp).		*P. erythroseptica.*	
	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L. t	W.	L.	W.	L.	W.
μ																								
19.5 to 21.49	.....	19	3	40	.....	1	.....	1	3	19	.....	4	....	82	12	64	16	71	1	44	.....	19	.....	5
21.5 to 23.49	2	75	7	78	.....	7	.....	3	9	45	.....	24	2	100	57	22	46	62	7	50	2	86	2	58
23.5 to 25.49	2	64	16	62	.....	8	.....	6	9	28	....	31	2	75	47	4	38	18	14	61	0	82	0	5
25.5 to 27.49	12	129	30	92	.....	44	3	29	20	46	2	79	4	32	111	16	75	10	26	92	11	120	12	119
27.5 to 29.49	10	47	37	33	.....	63	3	17	17	33	1	38	4	16	62	1	43	0	9	51	14	39	14	82
29.5 to 31.49	38	47	67	38	.....	80	5	39	51	53	8	79	6	15	50	1	56	1	35	40	42	16	0	0
31.5 to 33.49	27	7	45	14	.....	32	3	30	21	34	5	35	12	8	20	.....	24	.....	18	8	26	11	35	58
33.5 to 35.49	111	4	75	11	4	65	13	52	48	44	15	41	13	9	15	.....	28	.....	43	10	95	9	1	7
35.5 to 37.49	22	3	26	6	1	32	12	24	18	15	9	16	23	2	2	.....	16	.....	25	1	38	5	32	26
37.5 to 39.49	88	1	33	3	14	38	32	51	49	24	45	21	66	4	6	.....	16	.....	44	1	47	3	0	0
39.5 to 41.49	17	.....	6	0	12	17	11	21	26	15	13	7	32	0	4	.....	4	.....	20	0	30	1	38	12
41.5 to 43.49	33	.....	25	2	56	10	44	28	33	11	36	10	52	1	0	.....	4	.....	38	1	29	.....	50	2
43.5 to 45.49	15	....	8	.. ..	58	1	10	17	14	9	20	3	36	....	0	.....	1	.....	18	0	17	.....	6	0
45.5 to 47.49	8	....	6	.	44	0	40	23	38	3	69	1	45	....	0	.....	1	.....	33	0	8	.....	53	1
47.5 to 49.49	7	....	4	....	37	1	19	11	14	1	31	1	24	....	0	.....	.....	.....	[illegible]	0	10	.....	0	.....
49.5 to 51.49	6	.....	7	...	60	.	35	23	7	...	30	....	26	.....	1	.....	.....	.....	[illegible]	0	8	.....	58	.....
51.5 to 53.49	0	.....	2	.....	29		19	4	10	.....	19	.....	14	.....	.....	.....	.....	.....	6	0	9	.....	40	.....
53.5 to 55.49	0	.....	1	.....	38	. .	44	5	7	....	27	.. ..	18	....	.....	.....	.....	.....	10	0	5	.... ..	10	.....
55.5 to 57.49	0	.....	.....	.....	12	..	14	5	2	.....	8	.. ..	5	.....	.....	.....	.....	.....	2	1	3	.....	30	.....
57.5 to 59.49	1	.....	.....	.....	19	.....	30	4	2	.....	13	.....	5	.....	.....	.....	.....	.....	2	.....	2	.....	2	.....
59.5 to 61.49	0	.....	.....	.....	7	. .	13	1	0	.....	14	.....	2	.....	.....	.....	.....	.....	2	.....	3	.....	12	.....
61.5 to 63.49	1	.....	.....	.....	6	. .	19	.....	0	.....	11	.....	1	.....	.....	.....	.....	.....	5	.....	1	.....	0	.....
63.5 to 65.49	.....	.....	.....	.....	2	.	6	.....	1	.....	4	.....	0	.....	.....	.....	.....	.....	1	.....	.....	.....	2	.....
65.5 to 67.49	.....	.....	.....	.....	1	. .	13	.....	.....	.....	4	.....	0	.....	.....	.....	.....	.....	0	.....	.....	.....	2	.....
67.5 to 69.49	.....	.....	.....	.....	.....	. .	5	.....	.....	.....	3	.....	0	.....	.....	.....	.....	.....	1	.....	.....	.....	1	.....
69.5 to 71.49	.....	.....	.....	.....	.....	. .	3	.....	.....	.....	6	.....	2	.....	.....	.....	.....	.....	2	.....	.....	.....	.....	.....
71.5 to 73.49	.....	.....	.....	.....	.....	. .	1	.....	.....	.....	2	.....	0	.....	.....	.....	.....	.....	0	.....	.....	.....	.....	.....
73.5 to 75.49	.....	.....	.....	.....	.....	. .	2	.....	.....	.....	1	.....	6	.....	.....	.....	.....	.....	2	.....	.....	.....	.....	.....
75.5 to 77.49	.....	.....	.....	.....	.....	. .	1	.....	.....	.....	0	.....	.....	.....	.....	.....	.....	.....	0	.....	.....	.....	.....	.....
77.5 to 79.49	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1	.....	.....	.....	.....	.....	.....	.....	1	.....	.....	.....	.....	.....
79.5 to 81.49	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	0	.....	.....	.....	.....	.....	.....	.....	0	.....	.....	.....	.....	.....
81.5 to 83.49	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	0	.....	.....	.....	.....	.....	.....	.....	2	.....	.....	.....	.....	.....
83.5 to 85.49	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	0	.....	.....	.....	.....	.....	.....	.....	1	.....	.....	.....	.....	.....
85.5 to 87.49	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1	.....	.....	.....	.....	.....	.....	.....	2	.....	.....	.....	.....	.....
87.5 to 89.49	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2	.....	.....	.....	.....	.....	.....	.....	1	.....	.....	.....	.....	.....

In *P. cactorum* isolated from *Phyllocactus* sp. and from *Panax* sp. the greatest number of individuals as regards length and width fall in the same class, although the actual number of species in all the classes varies. The measurements of conidia of *P. fagi* show that it is closely related to *P. cactorum*. There is also a striking relationship between *P. infestans* and *P. phaseoli*.

As shown by the curves in figures 1 to 6, the classes containing the greatest number of individuals in the different species show the least variation for the width of the conidia. The length of the conidia therefore may be adopted arbitrarily to divide the species into two groups: Those in which the predominating class is more than 34.5 μ in length, and which include the forms *P. faberi*, *P. jatrophae*, *P. arecae*, *P. syringae*, and *P. erythroseptica*, and those in which the predominating class is 34.5 μ or less, and which includes the forms *P. cactorum*, *P. fagi*, *P. nicotianae*, *P. infestans*, and *P. phaseoli*.

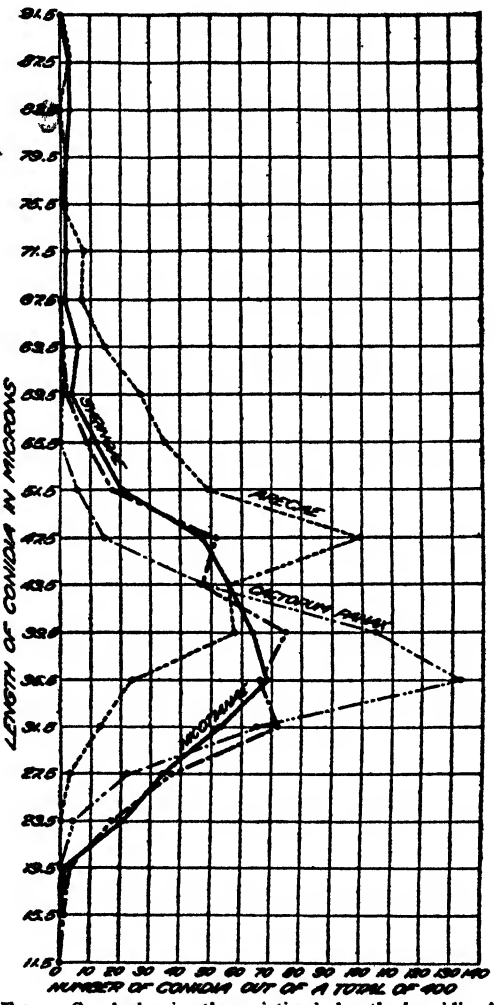

FIG. 3.—Graph showing the variation in length of conidia of *Phytophthora nicotianae*, *P. cactorum* (*Panax* sp.), *P. arecae*, and *P. syringae*.

Differences in length or width alone in different species do not give an accurate idea as to differences in form or shape of the conidia. The form or shape is a constant character and should be employed in differentiating the species. *P. parasitica*, for example, can be readily distinguished from *P. nicotianae* by the fact that the conidia of the former are long and elliptical, while those of the latter are ovate to globose, as shown in Plates 73 and 74. Similar differences exist in the conidia of other species also; but while such differences can be readily detected under the microscope, it has not been possible heretofore to express

them quantitatively. The writer proposes the following method for this purpose:

The differences ranging from ellipsoidal to spherical may be expressed largely as differences between the ratios of the length of the two diameters drawn perpendicular to each other. Thus, in text figure 7 is shown an extreme case of two forms, one ellipsoidal and the other spherical. The difference between these may be indicated by the difference between $\frac{aa}{bb}$ and $\frac{a'a'}{b'b'}$ and expressed quantitatively 2 and 1, since in this hypothetical case aa is twice the length of bb, and bb is equal to $a'a'$ and $b'b'$.

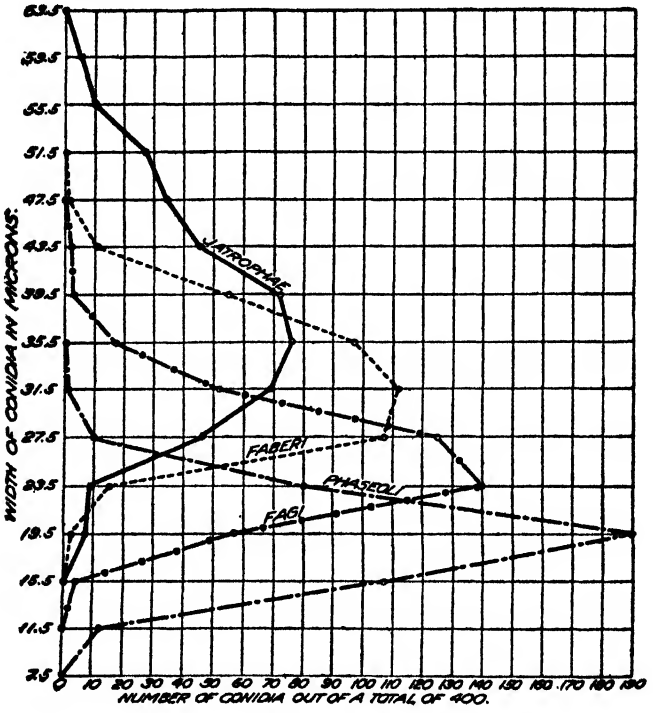

FIG. 4.—Graph showing the variation in width of conidia of *Phytophthora fagi*, *P. phaseoli*, *P. faberi*, and *P. jatrophae*.

As previously stated, the conidia of the *Phytophthora* spp. vary from elliptical to globose; therefore measurements made of each conidium of the long and short diameters, as shown above, will express quantitatively the difference heretofore expressed qualitatively. Such measurements in microns were made from conidia of each species taken at random from normal culture. Four hundred conidia were measured for each species and the ratio of the long and the short diameter of each determined. Figure 8 shows diagrammatically the classification of these ratios. It also shows the predominating ratio in each form, as well as the maximum and minimum limits. From this it will be seen at a glance that the

forms *erythroseptica, arecae,* and *parasitica* can be distinguished with ease from the remaining forms by the fact the former have a predominating ratio of more than 1.5 and the latter a predominating ratio of less than 1.5. Of the three forms having a ratio of more than 1.5, the predominating ratio of *P. parasitica* approaches 2, while that of *P. erythroseptica* and *P. arecae* is less than 1.75.

Another distinguishing character is the degree of development of the papilla of the conidia. Camera-lucida drawings of the papilla of typical conidia are shown in text figure 9. The papilla of *P. syringae* and *P. erythroseptica* are broad and flat and not easily distinguished from the curvature of the wall. The papilla of *P. faberi* are raised and very prominent, while the papilla of the other forms show all gradations between

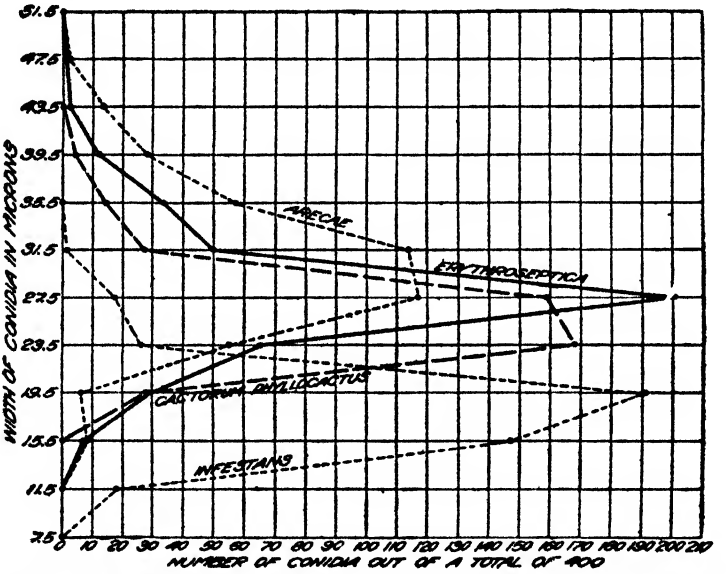

FIG. 5.—Graph showing the variation in width of *Phytophthora infestans, P. cactorum* (*Phyllocactus* sp.), *P. erythroseptica,* and *P. arecae.*

these two extremes. The shape of the papilla is constant under all conditions and therefore a good character for use in taxonomy.

GERMINATION OF THE CONIDIA.—In the 10 forms which the writer had in culture, the germination of the conidia was studied; and in all it was found that germination took place either by germ tube or swarm spores, as shown in Plate 75. Potentially each conidium is a sporangium, and, as regards the method by which it germinates, it is influenced, in part at least, by environment.

No differences were noted in the germination by means of germ tubes of the conidia from various species. The method may be described as follows: After the conidium is mature and the papillum formed, one or more tubes are produced. These arise from any point of the conidium,

but most generally near the apex of the spore. The contents of the conidium becomes less dense as a result of passing partly into the growing germ tubes. At the apex the germ tube usually rises from the side of the spore a short distance below the papillum, but seldom from the papillum itself, and in the case of many of these germinations it is very characteristic for the germ tubes to arise in a whorl or cluster near the papillum. As the tubes grow branches are formed.

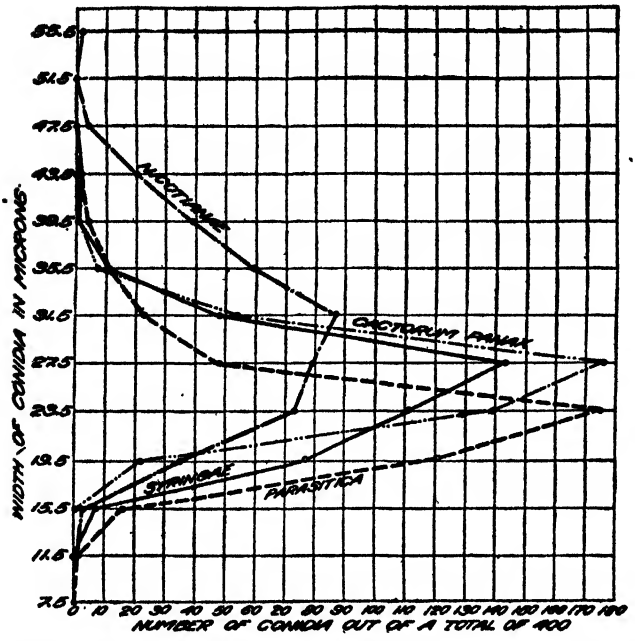

FIG. 6.—Graph showing the variation in width of *Phytophthora parasitica*, *P. syringae*, *P. cactorum* (*Panax* sp.), and *P. nicotianae*.

Swarm-spore germination is common not only to species of Phytophthora, but to some other members of the Peronosporineae. Detailed differences on which separation of genera in this group were based are supposed to exist. Speaking of the Peronosporineae, Butler (4) says:

> The genus Pythium is separated from all the rest by liberating its zoospores in an imperfectly differentiated state into a bladder at the mouth of the sporange, in which differentiation is completed. There are other minor differences, such as the aerial habitat and parasitism of the Peronosporacea, the formation of haustoria correlative with the latter, etc. None of these differences are absolute.

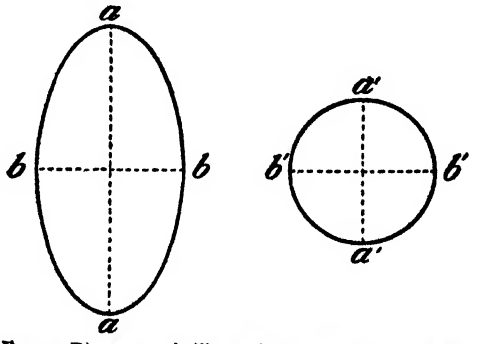

FIG. 7.—Diagrammatic illustration showing that the ratio of the long to the short diameter of ellipsoidal and spherical bodies offers a quantitative means for designating their shape.

In the study of swarm-spore germination in the genus Phytophthora it was found that at least some of the species liberate their zoospore mass into a bladder or vesicle similar to that described for *Pythium palmivorum* (Butler, 5). This shows a still greater relationship to *Pythium*

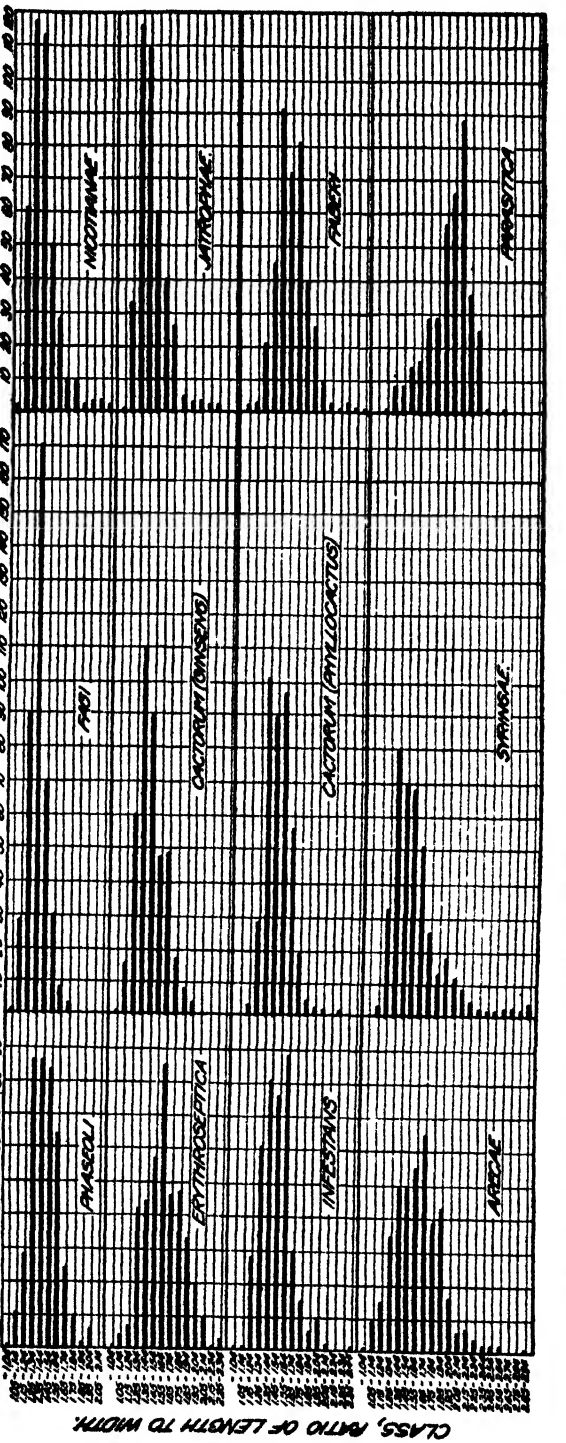

FIG. 8.—Diagram of the arrangement in classes of the ratios of the length to the width of the various conidia, showing the limits of variation and modes.

spp. than has been suspected. The writer observed this bladder-like membrane in *Phytophthora cactorum*, *P. arecae*, and *P. parasitica*. It was previously noted in *P. parasitica* by Dastur (10). Perhaps its evanescent nature explains why it had not been previously observed in some or in all the forms.

Considerable difficulty in obtaining swarm spores at will has been experienced by previous investigators. By means of the following-described method the writer at all times obtained good results: Cultures of the various species were made on media known to produce an abundance of conidia, 250 c. c. Erlenmeyer flasks being generally used for the purpose. When an abundance of conidia were obtained, a small quantity of sterile water was poured into the flask containing the culture, care being taken at the same time to prevent contamination. The flask was then gently shaken to dislodge the conidia from the conidiophores, and the suspension of the spores was poured into another sterilized Erlenmeyer flask and held at a temperature of about 15° C. At the end of from two to five hours a large majority of the conidia had germinated. By examining the solution at various intervals the different stages of germination, as shown in Plate 75, were readily seen. The length of time required for conidial germination depends on temperature conditions, as shown for *P. infestans* by Melhus (20), who found temperatures below 20° C. more favorable for indirect or zoospore germination than the higher temperatures. The age of the culture and the temperature at which spores are kept are the paramount conditions for germination by means of swarm spores.

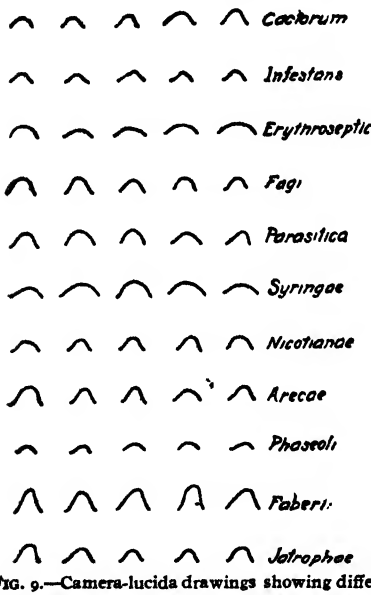

FIG. 9.—Camera-lucida drawings showing differences in the shape of the terminal papilla.

The first evidence of germination by swarm spores is the movement of protoplasmic granules in the sporangium. In the forms in which the vesicles occur the latter appear and enlarge gradually. Careful microscopic examination of a number of condia in different stages of development gives the impression that the terminal papillum enlarges and enters into the formation of the vesicle or that the membrane which lines the inner surface of the sporangium is stretched. The contents of the sporangium gradually pass into the vesicle, and after a time the latter ruptures and sets the swarm spores free. In many cases the vesicle and

its contents are much larger than the original sporangium, and it is difficult to understand how the large swarm-spore mass was contained in the smaller sporangium only a few seconds before. After the liberation of the swarm spores, the vesicles begin to contract, all signs of the opening disappear, and if any zoospores remain they are unable to escape. Plate 75 shows various stages in the germination of *P. arecae* illustrating these points.

When no vesicle is apparent, the process of swarm-spore germination is essentially the same as described, but there is in this case a variation in the manner of emergence of the swarm spores from individual conidia. From the narrow papilla-like opening the swarm spores issue singly or in pairs, the time of emergence varying from 4 to 10 seconds. At times the swarm spores are held together at the opening for 2 to 3 seconds, after which they swim in all directions. Frequently for some reason a few of the swarm spores do not emerge with the majority. For instance, in one case the writer observed some of these swarm spores struggle for 55 minutes to escape, but without success, although at times they were at the very opening. This gave the impression that the opening closed after the majority had escaped. In one particular case the swarm spores finally came to rest and germinated inside the sporangium, the germ tubes extending through the wall. In many cases two of the swarm spores after emergence seemed to stick together two or three seconds, being held by a fine protoplasmic thread or connection, and then each darted off by itself.

The number of swarm spores in a sporangium varies with the size of the latter, and the species are not characterized by a definite number. Neither do the swarm spores show any marked difference in the various species. All are pyriform to kidney-shaped, and each is provided with at least one light-colored spot, probably a vacuole, which is located near the concave side and which appears to be the point of attachment of two flagella. The latter are unequal in size and vary from one-half to twice the length of the body of the swarm spore. After swimming for a time, the flagella having disappeared, the spores finally rest, become round, and germinate by means of tubes. In none of the species of Phytophthora has diplanitism as occurring in related genera been found.

CHLAMYDOSPORES

The term "chlamydospore" is applied to one of the spore forms found in a number of species of Phytophthora. The chlamydospores are spherical, smooth, thick-walled, and brownish to yellow. They differ in size from the conidia and oospores and are formed either terminally or intercalarily. Various investigators have interpreted their function in different ways. When working with *P. faberi*, Von Faber (12), Coleman

(9), and Rorer (27) observed bodies which they took to be oospores. Von Faber states that he found oospores, but failed to find antheridia and oogonia; Coleman that the oospores "almost always fill the oogonial cavity so completely that the oogonial wall can be made out only with difficulty"; and Rorer refers to them as "parthenogenetic oospores."

In a pure culture of *P. faberi* originally obtained from Dr. Coleman an abundance of these bodies were produced. A great deal of time was devoted to their study in fresh as well as in sectioned and stained material. As a result of these studies the writer is convinced that they are not parthenogenetic oospores but are multinucleate vegetative bodies, serving the same function that chlamydospores serve in other groups of fungi. Dastur (10) observed similar bodies, which he termed "resting conidia," in *P. parasitica*, and pointed out that there is little probability of their being parthogenetic oospores. He says:

> They are over twice the size of normal oospores and that from the very beginning of their development they are thick-walled and slightly yellow tinted, while parthenogenetic oospores have their origin in thin-walled and hyaline oogonia, which on failing to come in contact with the antheridia after reaching maturity undergo the same change, at least outwardly, as they would have if they had been fertilized.

The chlamydospores were produced in culture in *P. faberi*, *P. parasitica*, *P. jatrophae*, and *P. nicotianae*. In all of these their general appearance is the same. Their contents are similar to that of the conidia, being granular, with one or more vacuoles, and are illustrated in Plates 76 and 77. It is significant that in all forms which produced an abundance of chlamydospores no oospores were produced in the cultures.

MEASUREMENTS OF CHLAMYDOSPORES.—Although similar in general appearance, the chlamydospores differ in size in the different species, and this difference is constant no matter on what media the culture is grown. As in the case of the conidia, great variation in size occurs within the species; hence, in order to determine the predominating size, as well as distribution in the various classes, 400 measurements of their diameters were made. The results are given in Table III. As will be seen from this table, the chlamydospores may vary in size from 17.5 to 59.5 μ, and *P. faberi* is the only one which has a predominating class of more than 32.5 μ. The difference in size of the predominating class of the chlamydospores of *P. faberi* and of the remaining three is sufficient to be employed in their separation.

TABLE III.—*Summary of measurements of chlamydospores and oospores of species of Phytophthora*

Class.	Chlamydospores.				Oospores.						
	P. faberi	*P. jatrophae*	*P. nicotianae*	*P. parasitica*	*P. cactorum* (*Phyllocactus* sp.)	*P. cactorum* (*Panax* sp.)	*P. fagi*	*P. syringae*	*P. phaseoli*	*P. arecae*	*P. erythroseptica*
μ											
17.5 to 19.49...	0	0	3	0	1	2	0	0	6	0	0
19.5 to 21.49...	0	0	18	0	5	3	2	0	16	0	0
21.5 to 23.49...	0	8	59	5	32	22	13	13	69	8	1
23.5 to 25.49...	0	16	45	17	58	41	14	23	92	9	3
25.5 to 27.49...	0	47	81	46	178	160	67	75	140	37	4
27.5 to 29.49...	4	45	40	58	68	95	72	87	47	45	9
29.5 to 31.49...	23	71	45	121	48	58	106	107	26	84	37
31.5 to 33.49...	25	41	28	59	7	12	51	53	3	67	44
33.5 to 35.49...	51	45	33	43	3	7	46	30	1	59	96
35.5 to 37.49...	52	37	5	20	0	0	13	6	0	37	61
37.5 to 39.49...	80	37	20	19	0	0	12	4	0	31	93
39.5 to 41.49...	45	25	4	6	0	0	2	1	0	8	29
41.5 to 43.49..	63	18	8	3	0	0	1	1	0	6	13
43.5 to 45.49...	19	6	3	2	0	0	0	0	0	6	8
45.5 to 47.49...	20	4	4	1	0	0	0	0	0	2	2
47.5 to 49.49...	11	0	3	0	0	0	0	0	0	1	0
49.5 to 51.49..	7	0	0	0	.....	.....	.....	.....	.....	.....	.....
51.5 to 53.49...	2	0	1	0	.....	.....	.....	.....	.....	.....	.....
53.5 to 55.49...	1	0	0	0	.....	.....	.....	.....	.....	.....	.....
55.5 to 57.49...	0	0	0	0	.....	.....	.....	.....	.....	.....	.....
57.5 to 59.49...	1	0	0	0	.....	.....	.....	.....	.....	.....	.....
Total.	400	400	400	400	400	400	400	400	400	400	400

The curves shown in figures 10 to 12 were constructed from the data given in Table III, except that the classes were made to differ by 4

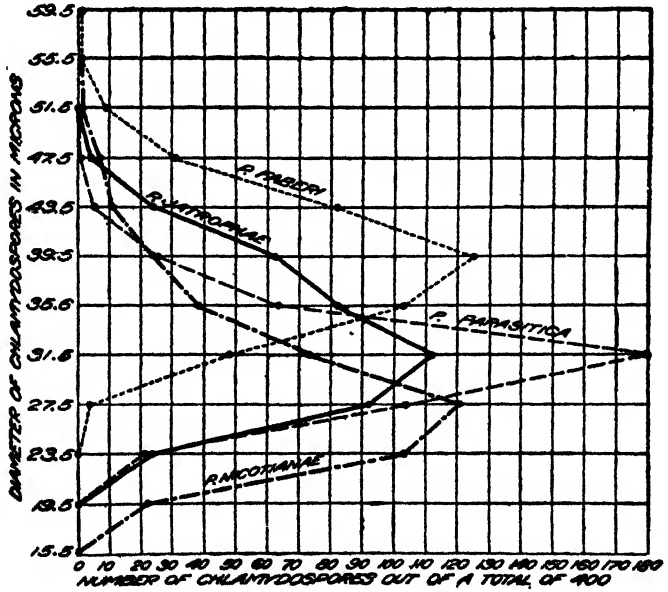

FIG. 10.—Graph showing the variation in the diameters of chlamydospores of *Phytophthora nicotianae*, *P. parasitica*, *P. faberi*, and *P. jatrophae*.

instead of 2 μ. Figure 10 shows the variation in the diameter of the chlamydospores in each of the species, as well as the differences in the

classes containing the greatest number of individuals in each species. Arranged in the order of size, beginning with the largest, we have *faberi*, *jatrophae*, *parasitica*, and *nicotianae*.

GERMINATION OF CHLAMYDOSPORES.—Dastur (10) states that the "resting" conidia do not require a period of rest before germination and that when grown in water germination takes place at the end of 24 hours. After a careful examination of the figures given by Dastur for the "resting" conidia of *P. parasitica* the writer has no doubt that they are identical with those which appeared in his own cultures and which are designated chlamydospores. So far, however, all attempts to germinate these either from young cultures or from cultures of considerable age have failed, as have also all attempts to germinate the chlamydo-

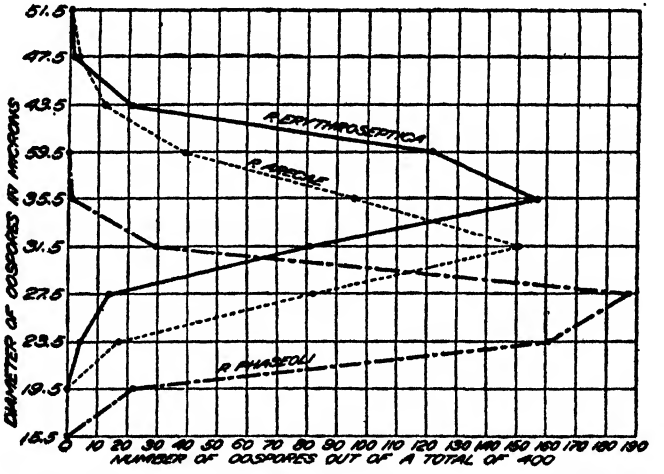

FIG. 11.—Graph showing the variation in the diameters of oospores of *Phytophthora phaseoli*, *P. arecae*, and *P. erythroseptica*.

spores of *P. nicotianae* and *P. jatrophae*. Recently the writer did obtain germination of chlamydospores in one of the species by wintering pure cultures, but the method of germination appeared to be so radically different from that observed by Dastur that it seems advisable to repeat the work before the results are presented to the public.

SEXUAL BODIES

Differences in the morphology of the sexual bodies in the different species of Phytophthora are the most constant characters under all conditions and therefore the most valuable for use in taxonomic work. External conditions, however, such as temperature, chemical nature of substrata, length of time a species has been in artificial culture, abundance of food supply, and may other factors have been known to influence the rapidity of development, as well as the number of sexual bodies produced in culture not only in the genus Phytophthora but in

other members of the Peronosporineae. For example, on its receipt from Dr. Coleman *P. arecae* had produced and for some time afterwards continued to produce an abundance of oospores in culture, but now, after remaining in culture for three years, it produces scarcely a single oospore. Dastur (10) observed this to be the case also in *P. parasitica*. He says: "There is as much variability and uncertainty in the production of these oospores as Clinton found in the production of *P. infestans*." Cultures of this species received by the writer from Dastur in 1914 and grown on media on which sexual bodies were previously reported by him failed to show a single oospore.

What actually determines the production of sexual bodies in *Phytophthora* spp. has not been solved. Some of the species produce these

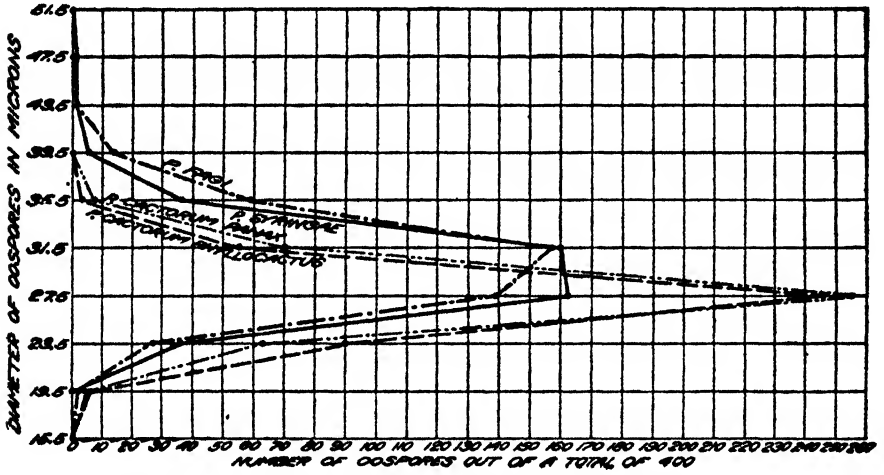

FIG. 11.—Graph showing the variation in the diameters of oospores of *Phytophthora cactorum* (*Phyllocactus* sp.), *P. cactorum* (*Panax* sp.), *P. fagi*, and *P. syringae*.

bodies readily and in great abundance on artificial media, while others produce few or none.

Oogonia and the subsequent development of oospores have been studied by the writer from cultures in *P. cactorum* from both *Phyllocactus* sp. and *Panax quinquefolia*, *P. fagi*, *P. syringae*, *P. phaseoli*, *P. arecae*, *P. erythroseptica*, and *P. infestans*. In addition *P. parasitica* Dastur (10), *P. nicotianae* von Breda de Haan (3), and *P. colocasiae* Butler and Kulkarni (6) are known to produce oospores in culture, but in the case of these three species oospores have not been seen by the writer.

As a result of four years of intermittent work with the various species of Phytophthora some general statements may be made on the behavior of these cultures as regards the development of the sexual bodies. The species *P. cactorum*, *P. fagi*, *P. phaseoli*, and *P. erythroseptica* produced oospores in great abundance on a number of media. In the case of *P. syringae*, *P. infestans*, and *P. arecae* their production requires special

media. Sterilized carrots were employed for *P. syringae*. A slight modification of oat agar as originally used—that is, Quaker-oat agar as used by Pethybridge and containing 7.5 gm. of agar to 1,000 c. c. of media—was employed for the remaining two.

The abundance of oogonia and oospores is so variable that this is a poor character for use in the separation of species. There is one exception, however—that is, *P. phaseoli* as compared with *P. infestans*. Although the former is very closely related to the latter, there is a striking contrast between them in the abundance of oospores produced on oat agar, a large number constantly developing in the case of the former and few or none in the case of the latter. The entire absence of oospores in *P. infestans*, or the scarcity in the development of them, has been observed by all previous investigators working with this species. Although it is not advisable to rely on this character alone in separating these species, it is useful for this purpose when taken in connection with more important characters.

Except in rare cases in which they arise intercalarily, the oogonia originate as terminal enlargements of the mycelial threads, as shown in Plates 76 and 77. In the former case it is often difficult, unless antheridia are present, to determine whether they are true oogonia or chlamydospores, as found in *P. faberi* (Pl. 77, *A*) and other species already mentioned. In certain forms the antheridium is formed first, and in others the oogonium; but the priority of development of these two organs is determined with such difficulty that it can not be used with safety in the separation of the species.

The position of the antheridium varies in the different species, and this variation is constant on all media and can thus be used in separating the genus into groups. These groups may be conveniently designated according to the specific name of one of the species belonging to each group, preferably the oldest described form, as first suggested by Pethybridge (24). The writer has established a third group, which embraces the forms in which the antheridia are entirely absent or in which the relation of the antheridium to the oogonium is unknown. This group is analogous to the Fungi Imperfecti. It will be seen, therefore, that there are three groups, which are designated the "*cactorum* group," the "*phaseoli* group," and the "*faberi* group."

CACTORUM GROUP.—In this group the antheridium, which is elliptical to reniform, is always distinct and is generally on the side of the oogonium. In some stages of development of the antheridium and oogonium a definite fertilization tube can be observed. After fertilization the antheridium disappears entirely or becomes transparent and the contents much reduced. *P. cactorum* (Pl. 77, *C*), *P. fagi* (Pl. 77, *D*), and *P. syringae* (Pl. 76, *A*) are included in this group, and according to Pethybridge probably *P. nicotianae* also. Fertilization in this group consists in a part

of the contents of the antheridium passing through a definite fertilization tube into the oogonium; and following its passage, the consequent changes in the growth of the oogonium into that of the oospore takes place.

PHASEOLI GROUP.—In this group the antheridium, which is not club-shaped, but approaches the form of a flattened sphere, is produced at the base of the oogonium; but no fertilization tube can be observed. After fertilization the antheridium remains attached to the oosphere, and there is very little, if any, change in its contents. In this group are included *P. phaseoli*, *P. arecae*, *P. erythroseptica*, *P. parasitica*, *P. colocasiae*, and *P. infestans* (?). These are shown in Plates 76 and 77. In reality this should be called the "*infestans* group," as *P. infestans* is the oldest described member, but in the opinion of the writer it has not yet been conclusively shown that the method of fertilization in *P. infestans* is the same as in the other members of the *phaseoli* group, and therefore it can logically be placed in the *faberi* group. In all the other members of this group the antheridium develops first and persists throughout the life of the oospore, which is not yet proved to be the case in *P. infestans*.

All previous investigators agree on the general absence of antheridia even in cultures showing all stages of development of the oospore-like bodies, and the author has confirmed their observations. In the few cases where antheridia are apparently present it is hardly safe to say what is the exact method of fertilization. For still other reasons given in subsequent paragraphs, it seemed best to designate this group as above, *P. phaseoli* being second as regards age since its description.

FABERI GROUP.—In this group the antheridia are entirely unknown either in artificial culture or on the natural hosts. It may also include forms in which if the antheridium is present its position, whether basal or side, is not known. It is a tentative group and contains forms which may be subsequently transferred to the other groups. As a result of the present study, it includes *P. faberi*, *P. jatrophae*, and *P. nicotianae*, as shown in Plates 76 and 77, the last form, according to Pethybridge, probably belonging to the *cactorum* group.

Until recently the only method of fertilization in species of Phytophthora was that found in the *cactorum* group, and, as explained, it consists of a fine light thread, or tube, sent out from the antheridium into the oogonium and through which a part of the contents of the former passes into the latter. Following this, the oogonium changes into an oosphere and finally into an oospore. In the forms placed in the *phaseoli* group, however, a new method of fertilization was recently observed. Clinton (7), in speaking of the antheridia and oogonia of *P. phaseoli*, writes:

> For a long time it was difficult to decide whether or not these threads did not actually penetrate the antheridium and grow through it, and we are not yet certain that this does not sometimes occur. Certainly the optical effect is frequently that of an internal thread, with its apical wall very thin as compared with the side walls. * * *. In

time, however, the oogonial thread reaches the top of the antheridium, and curving around its apex begins to swell into the oogonium, which by this time is usually cut off from its basal thread by a septum.

It was Pethybridge (24) who showed that the method of fertilization suggested by Clinton as possibly taking place in *P. phaseoli* actually did occur in *P. erythroseptica*. Dastur (10), working independently, showed that it also occurred in *P. parasitica*. It has since been noted by Butler and Kulkarni (6) in *P. colocasiae*, and Rosenbaum (28) has shown that *P. arecae* agrees with *P. erythroseptica* in this respect. So unique is this method of fertilization and the manner of formation of the antheridium and oogonium in the fungi that the details have not been worked out. Murphy (21), working with Pethybridge and later independently, has shown that a branch destined to become the oogonium grows through the antheridium and forms the oogonium after it emerges. Plate 76, *F*, shows several stages of this process in *P. phaseoli*. The antheridium continues to clasp permanently the stalk of the oogonium. According to Murphy (19), fertilization takes place when

a tube is pushed in from the antheridium through that part of the stalk of the oogonium which lies within the male organ, and through it a single male nucleus and the greater part of the cytoplasm passes in.

Following fertilization, the usual changes, which finally result in the oospore, take place.

In examining species belonging to the *cactorum* group a condition of the sexual organs similar to that found in the *phaseoli* group, which is brought about when the antheridium is superimposed on the stalk bearing the oogonium, is sometimes seen. This condition in *P. cactorum* is strikingly similar to that generally found in *P. arecae* and *P. phaseoli*, but is rare in the *cactorum* group.

Following the discovery of this unusual method of fertilization in some of the species of Phytopthora, Pethybridge (24) proposed the separation of the genus into two genera, the generic name Phytophthora to be retained for the group here designated as the *phaseoli* group, and "Nozemia," a new generic name, for the "*cactorum* group." In reviewing the genus, Wilson (37) suggested that if a new genus is to be established the name "Pleophytophthora," for reasons of priority, should be substituted for "Nozemia."

It does not seem to the writer that the genus Phytophthora should be subdivided into two genera at present, since both the genus Phytophthora and the genus Pythium, which are closely related, need to be investigated further. One of the main differences which has heretofore been supposed to exist between Phytophthora and Pythium is the production in the latter of a sacklike structure into which the swarm spores enter before being liberated. However, it has now been found that this structure is produced in several species of Phytophthora also;

and this, together with other characters, shows a very close relationship between these two genera. What constitutes a character of generic importance is, in the main, relative. It is of paramount importance that further study be made of the species belonging to Phytophthora and Pythium before new genera are established in these closely related groups.

SEXUAL BODIES OF P. INFESTANS

The question as to whether *P. infestans* produced oospores caused much discussion in earlier writings, and among some pathologists and mycologists the feeling that this question is not entirely solved still exists. A brief explanation of the statements regarding the oospore-like bodies of this species previously made in this paper will not be out of place here.

Among the number of strains of *P. infestans* which the writer grew on oat agar in the fall of 1912 one showed a noticeable number of brown, more or less globose bodies, which had every appearance of those described by Clinton (8) as the oospores of *P. infestans*. These differed from the oospores of the other species grown by the writer in that the antheridium was entirely absent in about 95 per cent of the cases examined, the remaining 5 per cent showing attachments which might or might not have been antheridia. These bodies were preserved in permanent mounts, and the many investigators to whom they were shown agreed that they were similar to the bodies described by Clinton and Pethybridge. The writer made diligent search for these bodies in transfers from several of the test tubes in which they appeared in 1912 and in other strains of this fungus, but without success until the fall of 1915, when he resorted to the following procedure:

Inoculations were made on the tops of healthy potato plants with cultures made from the strain of the fungus which originally produced oospores in culture in 1912. After infection took place and a crop of conidia was produced, the conidia were germinated and inoculations again made on potato foliage. In this way there were four separate inoculations and the culture was kept on the potato foliage for approximately a month. Following the last inoculation, the conidia were removed and placed in a small flask of sterilized water, which was kept at a temperature favorable for germination by means of swarm spores. When an abundance of these spores were produced, they were sprayed with a disinfected atomizer on raw potato blocks cut under sterile conditions, and within three days these blocks were covered with a heavy growth of the fungus. A large number of plantings were made from these to oat agar by means of a platinum needle. The first transfers were not free from bacteria, but by carefully watching the cultures and transferring at the proper time to other oat-agar slants a culture free from bacteria was finally obtained. On September 8, transfers from the culture which had recently been growing on

potato foliage were made to oat agar, and when examined 10 days later in a dozen test tubes oospore-like bodies, similar to those previously found by Clinton and Pethybridge, were found in each tube. Another transfer to oat agar from these test tubes produced a supply of oospore-like bodies, but subsequent transfers again gave negative results.

In a communication to the writer under date of June 25, 1915, Pethybridge states that "with *P. infestans*, however, the formation of sexual organs appears to stop after a time. I have not seen any in my cultures for many months now. The cultures, however, are still quite virulently pathogenic to potato tubers." The small number of oospores produced, together with the variability in their production, may explain the failure of so many investigators working with this species to observe these bodies even when grown on a medium favorable for their production.

In the case of *P. infestans*, which has been placed in the *phaseoli* group, Clinton (8) was unable to follow the different steps in the development of the oospores, owing to their scarcity, and claims that the oogonium is much more prominent than the antheridium, "since the latter is so frequently missing." Pethybridge, however, states unhesitatingly that the oospores of *P. infestans* follow the same course of development as in *P. erythroseptica*, and for this reason they are placed in the *phaseoli* group. In all the examinations made by the writer, however, only a few cases were found which might or might not be recognized as antheridia, and with the evidence at hand it does not seem to him that it has been conclusively proved that the oospores of *P. infestans* belong to the *phaseoli* group. On account of the scarcity of antheridia and indefinite knowledge as to their nature it would seem perhaps more logical at present to place them in the *faberi* group or the *phaseoli* group, bearing in mind that their relationship is not yet entirely solved. These are the reasons why the *phaseoli* group is not designated "*infestans* group," and they likewise constitute an additional argument against the subdivision of the genus at present into two genera.

Measurements of oospores.—Four hundred measurements were made of the diameter of the oospores of the various species, and, except in the case of *P. syringae*, cultures of which were made on sterilized carrot plugs, all were made from normal oat-agar cultures. Table III gives the results of these measurements, the first column showing the classes in microns and the other columns the number of oospores out of a total of 400 falling into each class. As it was impossible to obtain a sufficiently large number of oospores in the case of *P. infestans*, this species is not included in the table. The diameter of the oospores, as will be seen, varies from 17.5 to 49.5 μ. The greatest number of oospores of *P. cactorum* from *Phyllocactus* sp. and *Panax* sp. and of *P. phaseoli* fell in the same class—27.5 μ. It should be emphasized, however, that in the case of *P. phaseoli*, unlike that of *P. cactorum*, a larger percentage of the

oospores fall into the classes below 27.5 μ than into the classes above that number. The remaining species have slightly larger oospores.

The two curves in figures 11 and 12 show the diameter of the oospores of the species belonging to the *phaseoli* group, and, as in the case of the curve showing the measurements of the chlamydospores, were constructed from the data given in Table III and the classes made to differ by 4 instead of 2 μ. It will be seen that the oospores of *P. erythroseptica* have the greatest diameter and that those of *P. arecae* and *P. phaseoli* follow. The curves of the species of the *cactorum* group are shown in figure 12, but on account of the smaller variation the differences can not be used to advantage. In the case of *P. infestans* only 150 oospore-like bodies were measured. The results of the measurements of the diameters of these are shown in text figure 13. They varied from 23.5 to 39.5 μ, but most of them fell into the classes ranging from 38.5 to 44.5 μ.

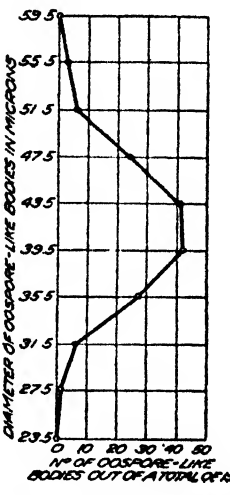

FIG. 13.—Graph showing the variation in the diameter measurements of the oospore-like bodies of *Phytophthora infestans*.

GERMINATION OF OOSPORES.—The methods followed in obtaining germination of oospores are given by the writer in another publication (29). To date *P. cactorum* from *Phyllocactus* sp., *P. cactorum* from *Panax* sp., and *P. fagi* from *Fagus* sp. have been germinated. The process of germination in these three forms is the same. After a period of rest and under proper conditions the oospore wall takes on a striated appearance, and later the striations disappear and the wall itself diminishes in thickness. Through a break in the wall a germ tube is sent out, and into this a part of the contents of the oospore gradually passes. When the tube has reached a certain size, it bears one or more conidia, and these also may germinate by means of germ tubes or by swarm spores.

BIOMETRICAL CONSTANTS

The use of biometry in obtaining biological data has been employed since the middle of the nineteenth century, but few biometrical studies have been attempted with fungi. On account of their variability, the spore forms of the genus Phytophthora offer a good object for such a study.

Pearl (22) says:

> A description which says nothing about the magnitude of the thing described is not complete, but on the contrary, lacks an element of primary importance * * * it is certain that not only are quantitative methods needed in biology, but also that a far more serious need is for something of the methodological viewpoint.

The biometrical methods therefore furnish a means of extending the descriptive method and of expressing quantitatively what investigators have heretofore attempted to express qualitatively.

The constants here obtained, offering a more ready comparison than the mass of individual measurements previously given, are (a) the mean or the point about which the individuals of the group cluster; (b) the median, the point on either side of which exactly half of the individuals fall; (c) the mode—that is, the class containing the greatest number of individuals; (d) standard deviation, expressing the degree of variation in the group; (e) skewness, which is a measure of "the degree of symmetry of the distribution of the individuals composing the group."

In the calculation of the constants the ordinary biometrical methods as given by E. Davenport (11) were followed, and for the moments Sheppard's corrections (33) were employed. In calculating the mode, median, skewness, and probable errors the formulæ given by Pearl and Surface (22) were used.

The writer is aware that the peculiar substrata on which some of the cultures were grown may affect the constants. It may be possible to give in a subsequent paper the results of measurements obtained under normal conditions—that is, directly from the host plants. The constants, together with their probable errors for the conidia of the various species, are shown in Table IV. In the examination of the table the following points should be noted: The species *P. cactorum* isolated from *Panax* sp. and *Phyllocactus* sp. show a remarkable uniformity. *P. fagi* is closely related to *P. cactorum*, the mean for length being slightly greater in the latter. The skewness for both length and width, as shown by *P. fagi*, is more closely related to the strain of *P. cactorum* obtained from *Panax* sp. than from *Phyllocactus* sp. *P. infestans* and *P. phaseoli* can not be distinguished by differences in size of the conidia. *P. jatrophae* and *P. nicotianae* are here shown to differ as regards both length and width. As previously pointed out, there are slight differences in the mycelium of the two species, the latter being somewhat broader and more gnarled and containing a larger amount of a substance of a fatty or glycogen nature. *P. infestans* and *P. erythroseptica*, both of which are parasitic on the potato (*Solanum tuberosum*), show great differences in size. The constants obtained for the standard deviation, as seen, show that the deviation from the mean for length varies from $4.31 \pm 0.102\ \mu$ in the case of *P. infestans* to as high as $11.10 \pm 0.264\ \mu$ in the case of *P. syringae*, and for width the deviation varies from $3.03 \pm 0.072\ \mu$ in the case of *P. infestans* to $8.27 \pm 0.197\ \mu$ in the case of *P. jatrophae*. Skewness in the case of length is positive in all cases, with the exception of *P. jatrophae*, *P. erythroseptica*, and *P. nicotianae*. In the case of width it is positive in all cases with the exception of *P. cactorum* (*Panax* sp.) and *P. syringae*.

Where the skewness is positive, the curve is more spreading on the side of large-sized individuals than in the opposite direction. In general this would point to the fact that the same conditions which favor the production of large individuals in the class as a whole is also more favorable to the production of exceptionally large individuals than exceptionally small ones.

TABLE IV.—*Constants for length and width of conidia of Phytophthora spp.*

LENGTH

Species.	Mean.	Median.	Mode.	Standard deviation.	Skewness (approximate).
	μ	μ	μ	μ	μ
P. fagi	33.65±0.210	33.19±0.264	32.28	6.23±0.148	0.0219±0.041
P. phaseoli	27.87± .195	27.31± .245	26.19	5.77± .137	.2912± .036
P. jatrophae	49.65± .328	49.79± .413	50.08	9.73± .232	−.0447± .041
P. cactorum (*Panax* sp.)	36.62± .181	35.46± .228	33.14	5.37± .128	.6478± .027
P. cactorum (*Phyllocactus* sp.)	37.69± .233	36.03± .293	32.70	6.91± .164	.7724± .025
P. erythroseptica	44.85± .303	45.88± .380	47.93	8.98± .214	−.3432± .035
P. arecae	47.92± .327	46.83± .411	44.65	9.70± .231	.3370± .035
P. syringae	39.86± .374	38.50± .470	35.79	11.10± .264	.3663± .035
P. infestans	27.08± .145	26.78± .183	26.19	4.31± .102	.2054± .039
P. parasitica	43.64± .271	42.88± .341	41.36	8.05± .191	.2833± .037
P. faberi	48.52± .198	48.09± .250	47.23	5.88± .140	.2193± .038
P. nicotianae	37.58± .274	37.62± .344	37.70	8.12± .193	−.0148± .041

WIDTH

P. fagi	25.67±0.146	25.47±0.185	25.08	4.33±0.103	0.1351±0.040
P. phaseoli	19.05± .105	18.86± .134	18.48	3.12± .075	.1826± .039
P. jatrophae	37.55± .279	36.92± .350	35.67	8.27± .197	.0227± .041
P. cactorum (*Panax* sp.)	26.06± .109	26.09± .139	26.16	3.24± .077	−.0324± .041
P. cactorum (*Phyllocactus* sp.)	25.72± .120	25.57± .153	25.27	3.57± .085	.1244± .040
P. erythroseptica	27.65± .177	27.16± .223	26.20	5.25± .125	.2750± .037
P. arecae	30.05± .186	29.85± .234	29.45	5.50± .131	.1090± .040
P. syringae	25.33± .152	25.61± .192	26.18	4.50± .107	−.1900± .039
P. infestans	18.27± .102	18.05± .130	17.61	3.03± .072	.2181± .038
P. parasitica	23.39± .147	22.74± .186	21.45	4.37± .104	.4428± .033
P. faberi	32.29± .159	31.40± .201	29.63	4.73± .113	.5615± .029
P. nicotianae	29.95± .230	29.84± .289	29.63	6.81± .162	.0463± .041

Means and standard deviation for the diameters of the chlamydospores and oospores are given in Table V. The smallest mean of the diameter of chlamydospores is that of *P. nicotianae*, with a standard deviation of $6.03 \pm 0.144\ \mu$, while *P. faberi* has the largest mean diameter, with a standard deviation of $4.97 \pm 0.118\ \mu$. *P. faberi* can well be separated from the three other forms producing chlamydospores by its larger chlamydospores. The differences in size of the oospores are less striking than those of the conidia, and the variations, as seen from the standard deviation, are likewise less. It has already been pointed out that *P. phaseoli*, *P. arecae*, and *P. erythroseptica* can be separated from the remaining forms in this table by morphological differences in the oospores, skewness being positive in all the forms with the exception of the oospores of *P. syringae*.

TABLE V.—*Constants for chlamydospores and oospores of species of Phytophthora*

Species.	Mean.	Median.	Mode.	Standard deviation.	Skewness.
	CHLAMYDOSPORES				
	μ	μ	μ	μ	μ
P. faberi	38.98±0.167	38.62±0.211	37.90	4.97±0.118	0.2173±0.0386
P. jatrophae	32.89± .180	32.13± .228	30.61	5.36± .128	.4257± .0332
P. nicotianae	28.83± .203	27.35± .256	24.39	6.03± .144	.7383± .0254
P. parasitica	31.15± .132	30.72± .167	29.86	3.92± .093	.3293± .0359
	OOSPORES				
P. cactorum (*Phyllocactus* sp.)	26.78±0.081	26.67±0.104	26.45	2.40±0.057	0.1374±0.0401
P. cactorum (*Panax* sp.)	27.36± .084	27.15± .108	26.73	2.50± .060	.2576± .0377
P. fagi	30.22± .125	30.10± .159	29.86	3.71± .088	.0970± .0407
P. syringae	29.50± .108	29.54± .137	29.62	3.19± .076	—.0376± .0412
P. phaseoli	25.55± .088	25.74± .113	26.12	2.60± .062	.2191± .0386
P. arecae	32.42± .152	32.01± .193	31.19	4.53± .108	.2751± .0374
P. erythroseptica	35.78± .127	35.70± .161	35.54	3.77± .090	.0636± .0410

The ratios of the length to the width of the 400 conidia measured are arranged into classes in figure 8. By the use of these data, representing 400 ratios of length to width of conidia, the means and the standard deviation of the ratios for each species were determined. The results are shown in Table VI. From this it will be clearly seen that the ratio of the length to the width of the conidia can be made use of in the separation of the species; and, in fact, it is suggested that a similar system of measurements in other fungi may perhaps be made use of in delineating species.

TABLE VI.—*Constants as calculated from class ratios of length to width of 400 conidia of species of Phytophthora as shown in figure 9*

Species.	Means.	Standard deviation.	Species.	Means.	Standard deviation.
	μ	μ		μ	μ
P. fagi	1.25±0.004	0.110±0.003	*P. infestans*	1.45±0.006	0.189±0.005
P. phaseoli	1.40± .006	.170± .004	*P. parasitica*	1.82± .009	.259± .006
P. jatrophae	1.28± .006	.168± .004	*P. cactorum* (*Phyllocactus* sp.)	1.39± .005	.149± .004
P. cactorum (*Panax* sp.)	1.34± .006	.165± .004	*P. faberi*	1.47± .007	.197± .005
P. erythroseptica	1.57± .008	.229± .005	*P. nicotianae*	1.25± .006	.171± .004
P. arecae	1.59± .009	.278± .007			
P. syringae	1.49± .011	.314± .007			

Reitz and Smith (26) make the following statement regarding the significance of probable errors:

In the comparison of two statistical results, the difference between the two results compared to its probable error is of great value. In general, we may take the probable error in a difference to be the square root of the sum of the squares of the probable errors of the two results.

If the difference does not exceed two or three times the probable error thus obtained, the difference may reasonably be attributed to random sampling. If the difference between the two results is as much as five to ten times the probable error, the probability of such differences in random sampling is so small that we are justified in saying that the difference is significant. In fact, a difference of ten times its probable error is certainly significant in so far as there is certainty in human affairs.

TABLE VII.—*Summary of differences in the means of the conidia of species of Phytophthora*

Species.	Difference in means.		Difference in means divided by probable error of difference.	
	Length.	Width.	Length.	Width.
	μ	μ	μ	μ
P. nicotianae and *P. jatrophae*	12.07±0.427	7.60±0.361	28.27	21.05
P. parasitica and *P. arecae*	4.28± .425	6.66± .237	10.07	28.10
P. fagi and *P. cactorum* (*Phyllocactus* sp.)	4.04± .374	.05± .188	12.87	.26
P. infestans and *P. erythroseptica*	17.77± .336	9.38± .204	52.89	45.98
P. infestans and *P. phaseoli*	.79± .243	.78± .146	3.25	5.34
P. cactorum (*Panax* sp.) and *P. cactorum* (*Phyllocactus* sp.)	1.07± .295	.34± .162	3.63	2.10
P. parasitica and *P. erythroseptica*	1.21± .407	4.26± .230	2.97	18.52
P. arecae and *P. erythroseptica*	3.07± .446	2.40± .257	6.88	9.34
P. syringae and *P. cactorum* (*Phyllocactus* sp.)	2.17± .441	.39± .194	4.92	2.01

The probable errors in the differences of the statistical results are shown in Tables VII and VIII. Examination of these tables shows that the differences in length of conidia of *P. nicotianae* and *P. jatrophae*, *P. parasitica* and *P. arecae*, *P. fagi* and *P. cactorum*, and *P. infestans* and *P. erythroseptica* are, without doubt, significant. On the other hand, the figures confirm the previous observation and statement that the pairs *P. infestans* and *P. phaseoli*, *P. cactorum* from *Panax* sp. and *P. cactorum* from *Phyllocactus* sp., *P. parasitica* and *P. erythroseptica* can not be distinguished by their differences in length.

TABLE VIII.—*Summary of differences in the mean diameter of oospores and chlamydospores of species of Phytophthora*

OOSPORES

Form.	Difference in mean diameter	Difference in mean diameter divided by probable error of difference.
	μ	μ
Arecae and *erythroseptica*	3.35±0 198	16.92
Cactorum (*Panax* sp.) and *cactorum* (*Phyllocactus* sp.)	.59± .117	5.04
Phaseoli and *arecae*	6.87± .176	38 92
Fagi and *cactorum* (*Phyllocactus* sp.)	3.44± .149	23.09
Syringae and *cactorum* (*Phyllocactus* sp.)	2.72± .135	20.15

CHLAMYDOSPORES

Nicotianae and *jatrophae*	4.05± .272	14.89
Jatrophae and *faberi*	6.09± .246	24.76
Faberi and *parasitica*	7.83± .209	37.46

The difference between the pairs *P. arecae* and *P. erythroseptica* and *P. syringae* and *P. cactorum* may or may not be due to random sampling. As regards width, *P. nicotinae* and *P. jatrophae*, *P. parasitica* and *P. arecae*, *P. parasitica* and *P. erythroseptica*, and *P. infestans* and *P. erythroseptica* are certainly different; differences which may or may not be significant are found between *P. infestans* and *P. phaseoli*, and *P. arecae* and *P. erythroseptica;* and finally the differences between *P. cactorum* from *Panax* sp. and *P. cactorum* from *Phyllocactus* sp., *P. fagi* and *P. cactorum*, and *P. syringae* and *P. cactorum* are of no value.

A study of Table VIII shows the differences in the means of the oospores of the various species to be significant and also confirms the identity of *P. cactorum* from *Panax* sp. and from *Phyllocactus* sp. The differences in the mean diameter of the chlamydospores, as given in this table, are likewise significant.

IDENTIFICATION AND SYSTEMATIC SEPARATION OF SPECIES

In determining a species belonging to the genus Phytophthora, which was one of the objects of this study, the following points should be noted, and it is suggested that as many of these as possible be used in the descriptions:

(1) Relation of antheridium to oogonium, whether basal or side.

(2) Shape of terminal papilla and their relation to the curvature of the body.

(3) Measurements of length and width of conidia, and the diameter of chlamydospores and oospores (at least 200 individuals should be measured).

(4) Arrangement of these measurements into classes and determination of the various biometrical constants.

(5) Calculations of the mean length and width of conidia and mean diameter of chlamydospores and oospores.

(6) Ratios of length to width of conidia measured and arrangement of these into classes, showing the mode.

(7) Mean, as determined from ratios of length and width.

(8) Character of conidiophores.

(9) Germination of conidia, character of swarm spore, etc.

(10) Growth on various media at room temperature, and noting macroscopic appearance and spore forms produced at the end of two and six weeks.

(11) Miscellaneous, such as peculiarities in mycelium.

It should be borne in mind that no definite criteria are known for the identification of species of some of the genera closely related to Phytophthora, among which may be mentioned Pythium, Pythiacystis, Peronospora, Plasmopara, and Sclerospora. Until further studies have been made, however, the above points can be employed to good advantage in identifying the species of these genera also.

The following tentative table is offered for the separation of species:

A. CACTORUM GROUP.—Majority of the oogonia have the antheridium on side. *P. cactorum*, *P. fagi*, *P. syringae*, *P. nicotianae*.

B. Apical papilla not prominent, but broad and flat.

C. Mean length of conidia 39.86 μ, mean width 25.33 μ, mode for width 26.18 μ, mean diameter of oospore 29.5 μ..... 1. *P. syringae*.

BB. Apical papilla prominent.

C. Chlamydospores absent, mode for length of conidia 32 to 33 μ, mode for width of conidia 25 to 26 μ.

D. Mode for diameter of oospores 29.86 μ, mean diameter 30.22 μ..... 2. *P. fagi*.

DD. Mode for diameter of oospores 26 μ, mean diameter 27 μ.....3. *P. cactorum*.

CC. Chlamydospores produced abundantly, mode for length of conidia 37 to 38 μ, mode for width 29 to 30 μ.

D. Mode for diameter of chlamydospores 28.86 μ, mean diameter 31.15 μ, mean ratio of length to width of conidia 1.25.....4. *P. nicotianae*.

AA. PHASEOLI GROUP.—Majority of oogonia have the antheridium at the base. *P. phaseoli*, *P. erythroseptica*, *P. arecae*, *P. parasitica*, and *P. infestans*.

B. Mean ratio of length to width of conidia greater than 1.75.

C. Mean length of conidia 43.64 μ, mean width 23.39 μ, mean diameter of chlamydospores 31.15 μ... 5. *P. parasitica*.

BB. Mean ratio of length to width of conidia less than 1.75.

C. Conidia small, mean length less than 30 μ, mean width less than 20 μ.

D. Producing an abundance of oospores on oat agar, antheridia present..... 6. *P. phaseoli*.

DD. Oospores or oospore-like bodies lacking or very scarce on oat agar and when present having antheridia entirely absent or indefinite. 7. *P. infestans*.

CC. Conidia large, mean length more than 30 μ, mean width more than 20 μ.

D. Apical papilla not prominent, but broad and flat.

E. Mean diameter of oospore 35.78 μ with a standard deviation of 3.77, mode for length of conidia 47.93 μ, mode for width of conidia 26.20 μ.....8. *P. erythroseptica*.

DD. Apical papilla not as above but prominent, mean diameter of oospore 32.42 μ with a standard deviation of 4.53, mode for length of conidia 44.65 μ, mode for width of conidia 29.45 μ. 9. *P. arecae*.

AAA. FABERI GROUP.—Antheridium entirely unknown or its relation to the oogonium not yet determined, chlamydospores absent or present. *P. faberi*, *P. jatrophae*.

B. Chlamydospores large, mean diameter more than 35 μ.

C. Mean diameter of chlamydospores 38.98 μ, mean ratio of length to width of conidia 1.47.....10. *P. faberi*.

BB. Chlamydospores small, mean diameter less than 35 μ.

C. Mean diameter of chlamydospores 32.89 μ, mean ratio of length to width of conidia 1.28.....11. *P. jatrophae*.

SUMMARY

Prior to these studies no definite criteria for the identification and separation of the species belonging to the genus Phytophthora were known. With a view to supplying information for this purpose, 11 out of 13 described species were collected and studied from pure cultures grown on artificial media and from herbarium material. The results of these studies may be summarized as follows:

(1) As regards rate of growth and spore forms produced, the various species reacted differently on the different media.

(2) The temperature at which cultures are grown is a factor in the production of normal and comparable cultures.

(3) For the purpose of testing the purity of the cultures from time to time two methods the details of which are described on pages 236–237 were devised.

(4) Continual culturing from large selected conidia does not eventually produce a culture with a predominance of large individuals, or vice versa.

(5) The separation and relationship of species should be made on the aggregate of characters, it being borne in mind that the proportionate value to be attached to each character must necessarily vary.

(6) As a minor character, the macroscopic growth on a given medium is of some value.

(7) The time of appearance of the spore forms from different strains of the same species on a given medium may vary, but eventually the same forms appear.

(8) On oat agar the mycelia of the various species can not be distinguished with any degree of certainty. On potato agar *P. syringae* can be distinguished from the remaining species by the fact that it produces characteristic tuberculate mycelia (Pl. 71, *A*); and likewise *P. nicotianae* can, to a certain extent, be distinguished by the more gnarled mycelia and greater abundance of globoid particles of a fatty or glycogen nature within the threads.

(9) Measurements of the conidia can be employed as an aid in delineating species provided a sufficiently large number are measured. It is suggested that at least 200 should be measured and the different measurements grouped into classes.

(10) For the purpose of obtaining a quantitative measure of the shape of the conidia the ratio of the length to the width should be ascertained and the ratios likewise grouped into classes. A comparison of the conidia of *P. parasitica* and *P. nicotianae* illustrated this point. Heretofore the differences in shape of the conidia of these two species would be expressed only qualitatively, the former being called long and ellipsoidal and the latter short and globose. As a result of measuring the length and width of 400 individuals of each species and obtaining the ratios of the length to the width, the conidia of *P. parasitica*, expressed quanti-

tatively, group themselves about a predominating ratio of 2, while those of *P. nicotianae* are found at 1.2 (fig. 8). Similarly the ratios of the conidia of all the remaining species vary from 1 plus to 2 plus (fig. 8).

(11) The degree of development of the papillum is a good character to be employed in taxonomic work (fig. 9).

(12) In their germination of conidia by means of swarm spores at least some of the species of Phytophthora liberate their zoospore mass into a bladder or vesicle, thus showing a greater relationship to *Pythium* spp. than had been suspected (Pl. 75).

(13) Certain species of Phytophthora produce chlamydospores either terminally or intercalarily.

(14) The measurements of the chlamydospores can be used to good advantage in delineating species. A sufficiently large number should be measured, as in the case of the conidia.

(15) The relation of the antheridium to the oogonium—that is, whether produced at the base or on a side—can be used in separating the genus into groups.

(16) An additional group (*faberi* group), analogous to the Fungi Imperfecti group and embracing forms in which the antheridia are absent or their relation to the oogonium yet unknown, is tentatively established.

(17) In one of the several strains of *P. infestans*, oospore-like bodies, resembling those observed by other investigators, were produced, but antheridia were absent or, if present, were of a doubtful nature.

(18) As in the case of conidia and chlamydospores, a sufficiently large number of measurements of the oospores should be made if the measurements are to be used in identifying a species.

(19) On account of the variability in the size of the spore forms belonging to the genus, a more ready comparison can be made by the use of biometrical constants than by a mass of individual measurements, and such biometrical constants were calculated and arranged into tables. By the use of these constants the identity and relationship of the species are further confirmed.

(20) There being no known criteria for the separation and identification of species in closely related genera, such as Pythium, Pythiacystis, Peronospora, Plasmopara, and Sclerospora, most of the points enumerated in this paper can be employed to good advantage for this purpose in connection with these genera.

(21) A tentative table, employing some of the above characters for the separation of the species, is given on page 271.

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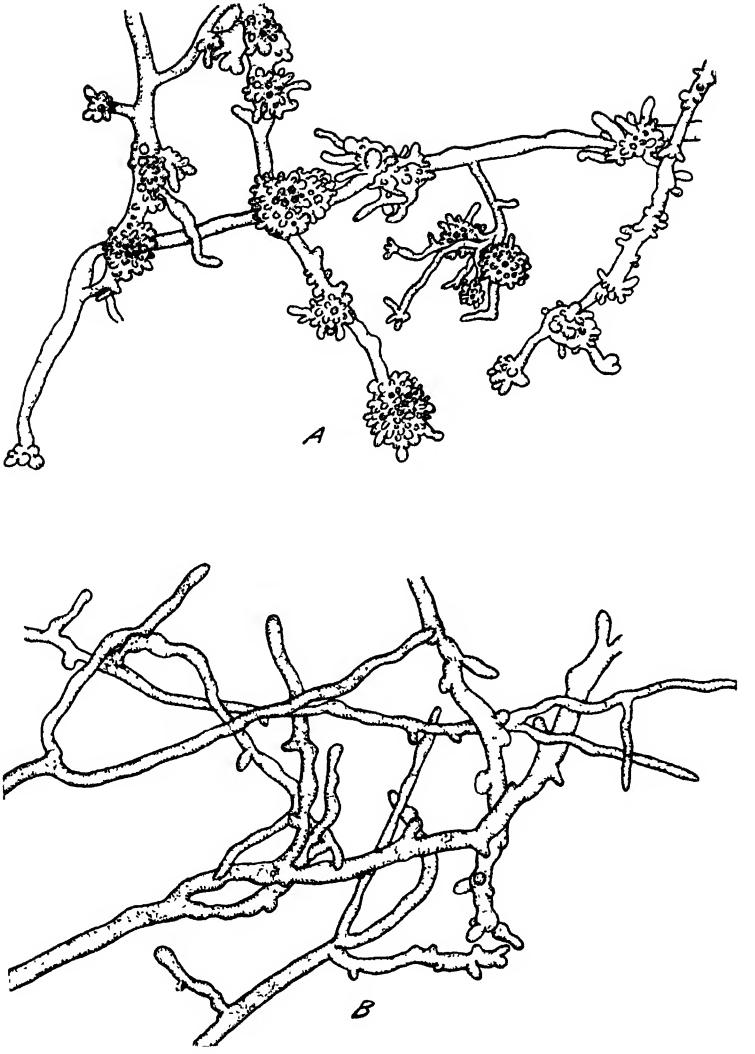
PLATE 71

A.—Mycelium of *P. syringae* grown on potato agar. Note the tuberculate appearance of the mycelium. ×320.

B.—Mycelium of *P. syringae* grown on oat agar This is the general appearance of the mycelia of the other forms when grown on this medium. ×320.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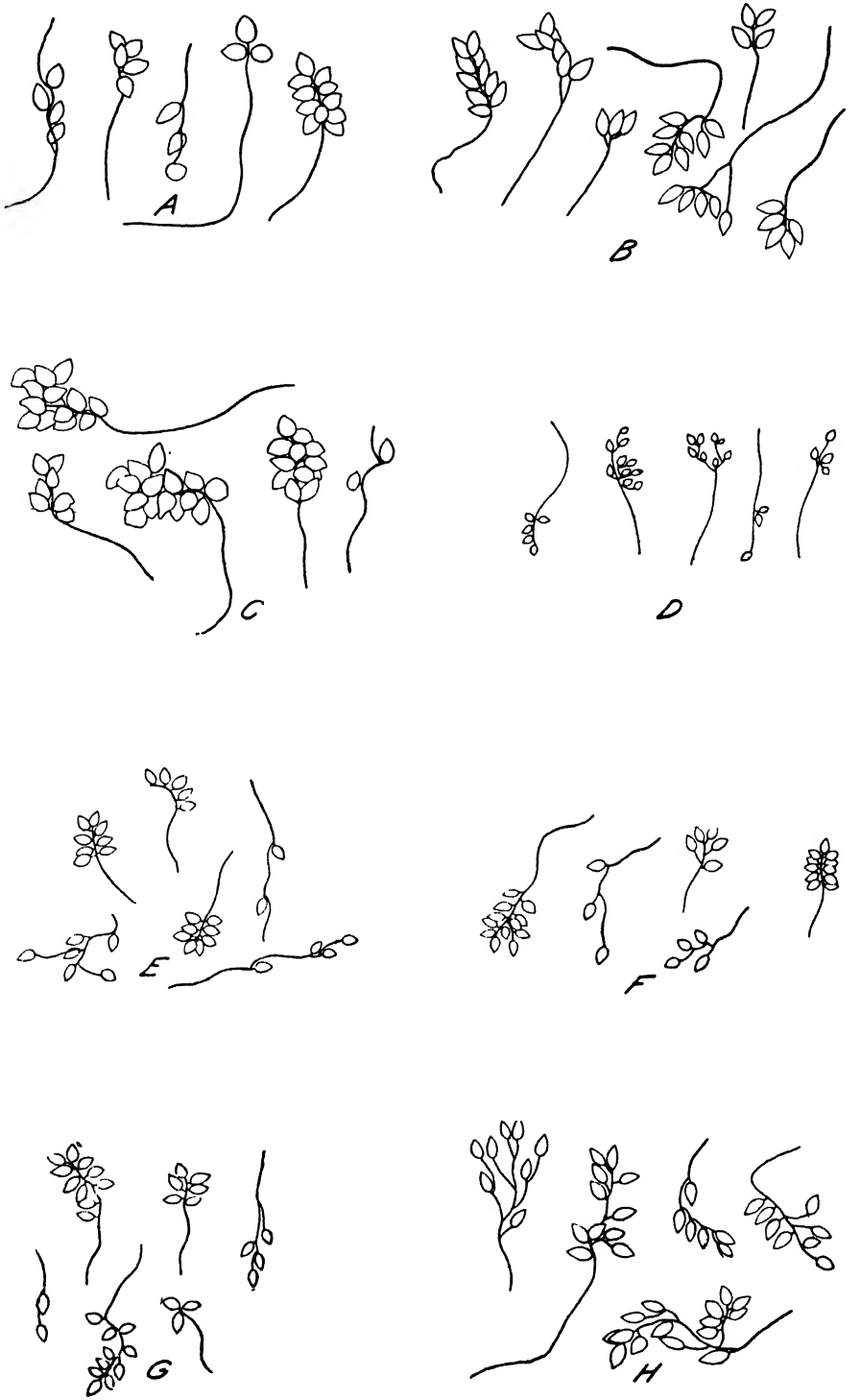
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PLATE 72

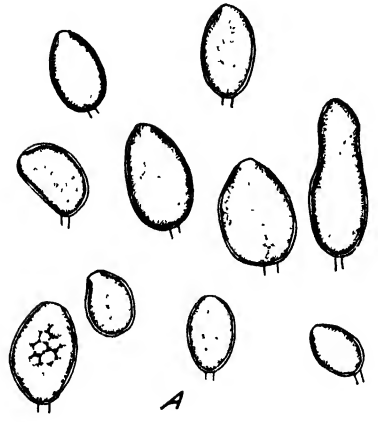
Conidiophores of the various species of Phytophthora studied from Van Tieghem cells. ×54.

A.—*P. jatrophae.*
B.—*P. arecae.*
C.—*P. nicotianae.*
D.—*P. phaseoli.*
E.—*P. cactorum* (*Panax* sp.).
F.—*P. fagi.*
G.—*P. cactorum* (*Phyllocactus* sp.).
H.—*P. faberi*

PLATE 73

Conidia of the various species of Phytophthora illustrating differences in shape of conidia and appearance of the terminal papilla. ×320.

A.—*P. erythroseptica.*
B.—*P. fagi.*
C.—*P. arecae.*
D.—*P. phaseoli.*
E.—*P. parasitica.*
F.—*P. infestans.*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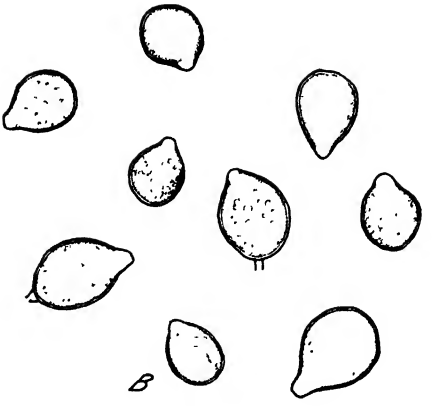
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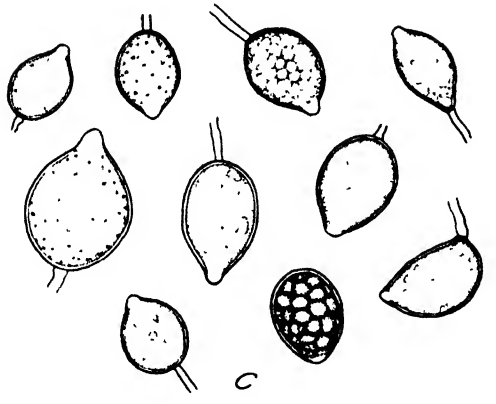
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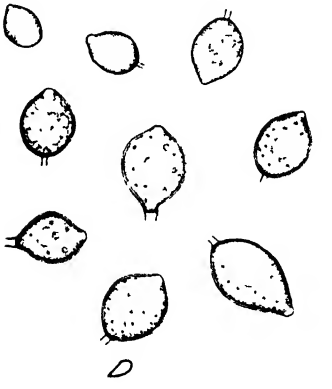
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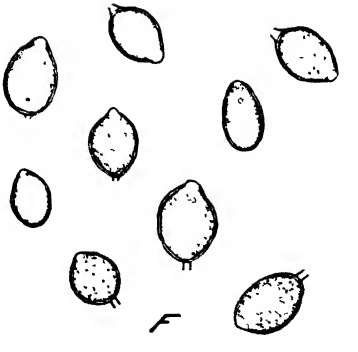
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PLATE 74

Conidia of the various species of Phytophthora illustrating differences in shape of conidia and appearance of the terminal papilla. ×320.

A.—*P. cactorum* (*Phyllocactus* sp.).

B.—*P. syringae.*

C.—*P. faberi.*

D.—*P. nicotianae.*

E.—*P. jatrophae.*

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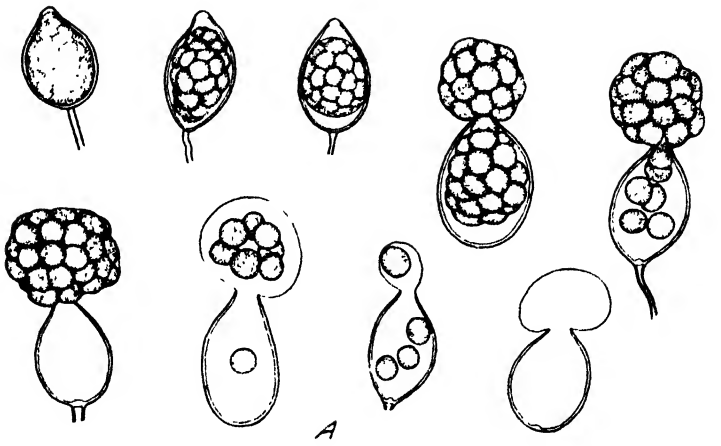
PLATE 75

Stages in the germination of the conidia and swarm spores of *P. arecae*. ×320.
A.—Earlier stages.
B.—Later stages, showing the escape of the swarm spores and their germination.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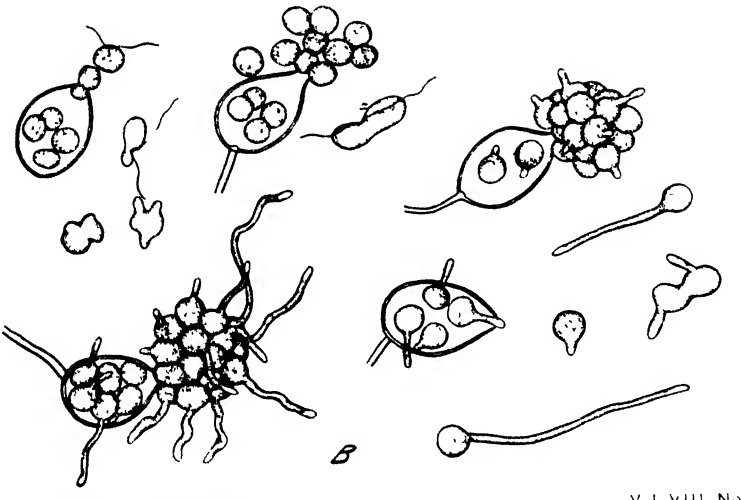
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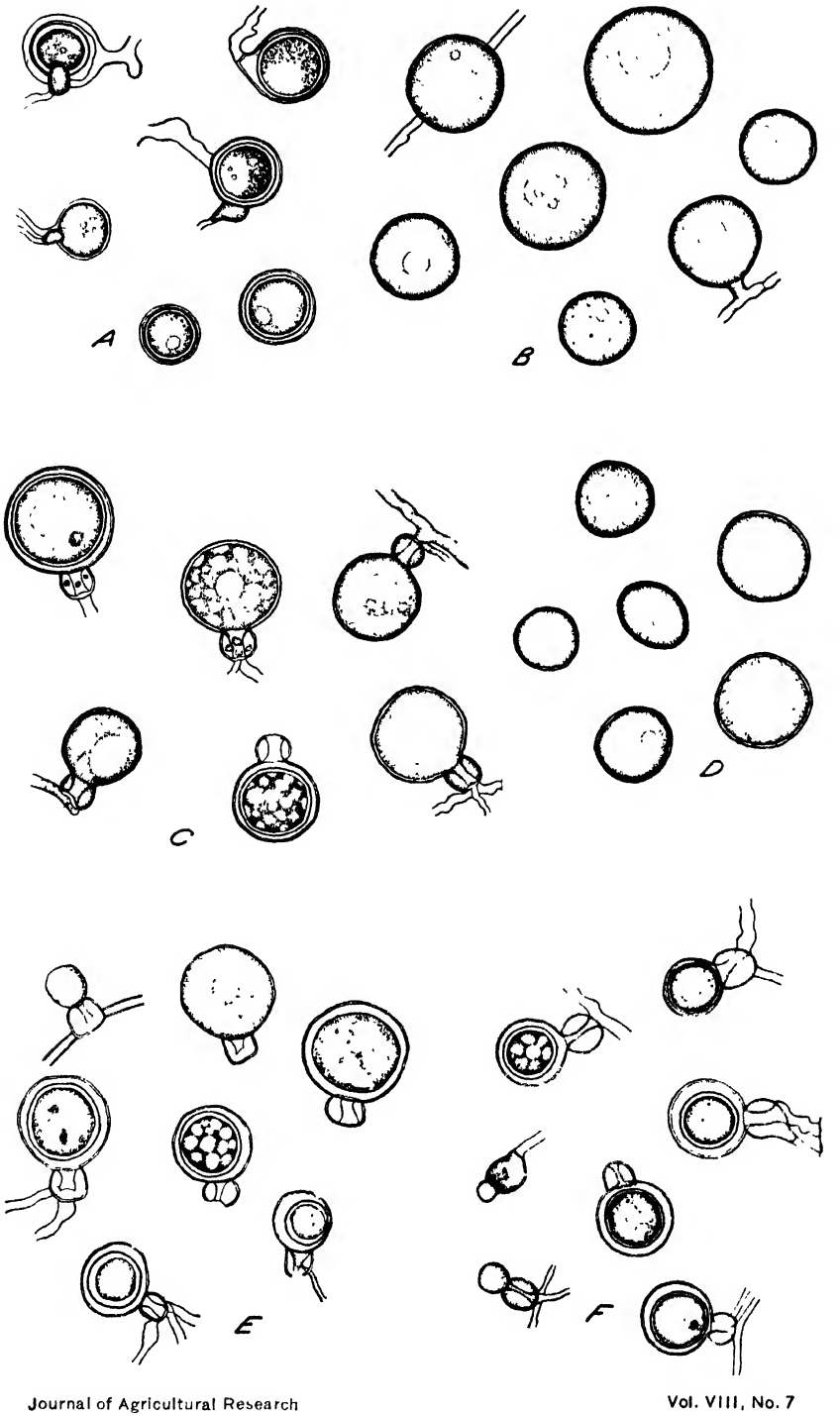
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PLATE 76

Chlamydospores and oospores of the various species of Phytophthora, illustrating shape and relation of the antheridium to the oogonium. ×320.

A.—Oospores of *P. syringae.*

B.—Chlamydospores of *P. jatrophae.*

C.—Oospores of *P. arecae.*

D.—Chlamydospores of *P. parasitica.*

E.—Oospores of *P. erythroseptica.*

F.—Oospores of *P. phaseoli.*

PLATE 77

Chlamydospores and oospores of the various species of Phytophthora, illustrating shape and relation of the antheridium to the oogonium. ×320.

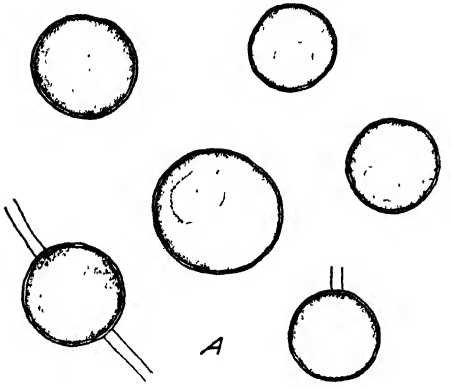
A.—Chlamydospores of *P. faberi.*

B.—Oospore-like bodies of *P. infestans.*

C.—Oospores of *P cactorum* (*Phyllocactus* sp.).

D.—Oospores of *P. fagi.*

E.—Chlamydospores of *P. nicotianae.*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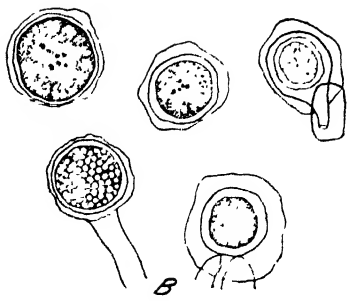
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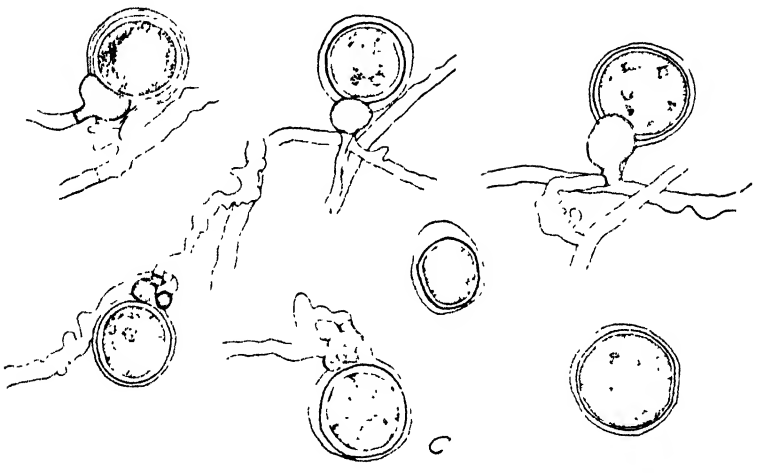
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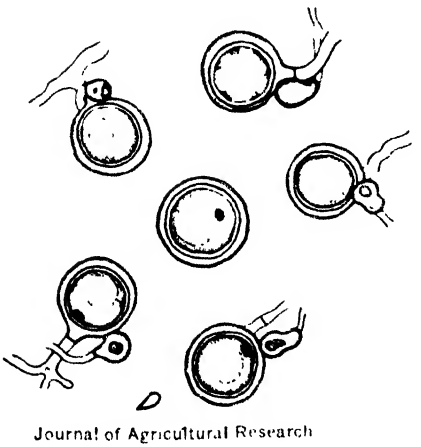
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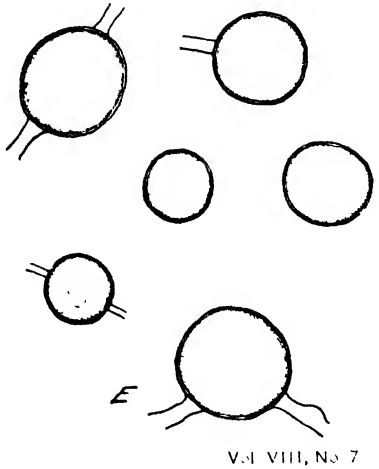
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TETRASTICHUS BRUCHOPHAGI, A RECENTLY DESCRIBED PARASITE OF BRUCHOPHAGUS FUNEBRIS

By THEODORE D. URBAHNS,

Entomological Assistant, Cereal and Forage Insect Investigations, Bureau of Entomology, United States Department of Agriculture

INTRODUCTION

Tetrastichus bruchophagi Gahan was one of several new species of parasites found attacking *Bruchophagus funebris* Howard, which was breeding in alfalfa seeds throughout the San Joaquin and other valleys of California during the seasons of 1912 and 1913. Infested alfalfa seeds (*Medicago sativa*) were obtained at different times from various localities, and these showed that this parasite attacked *B. funebris* over a widely distributed area.

Most of the observations concerning the habits of this parasite were made from material taken to the temporary laboratory at Glendale, Cal.

DISCOVERY OF THE INSECT AND ITS DISTRIBUTION

This species was first reared by the author in November, 1912, from *B. funebris* infesting alfalfa seeds which were collected at Tulare, Cal. On April 22, 1913, it was secured from material collected at Corcoran, Cal., and on July 9, from Glendale, Cal. It was reared on August 12 of the same year from Yuma, Ariz.; on August 26 from Sacramento, Cal.; and on October 11 from Dos Palos, Cal. During 1914 it was also reared, on June 16, from Stockton, Cal.; September 8, from Red Bluff, Cal.; and September 16, from Woodland, Cal., from alfalfa seeds collected at these points.

On September 11 it was reared from *B. funebris* infesting red-clover seeds (*Trifolium incarnatum*) taken at Albany, Oreg. During April, 1915, it was found on *B. funebris* in alfalfa seeds from Bishop, Cal., and was reared from red-clover heads infested by *B. funebris*, secured through Mr. W. J. Phillips at Charlottesville, Va. On June 10 it was reared from infested red-clover heads secured from Mr. J. J. Davis at La Fayette, Ind., and on July 8 it was reared from red-clover heads secured from Akron, Ind. Mr. C. N. Ainslie reared miscellaneous insects from red clover collected at Hudson, Mich., August 21, 1906, and from Rochester, Minn., August, 1907, among which this species was found.

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Adults of a species of Tetrastichus, of which the specific determination was not given, have been reported as having been reared, together with *B. funebris*, from red clover collected as follows:

E. G. Titus, March 20, 1905, Daggett, Mich.
F. M. Webster, May 24, 1906, Chambersburg, Pa.
F. M. Webster, May 24, 1906, Milton, Pa.
F. M. Webster, January 24, 1906, State College, Pa.
F. M. Webster, January 31, 1906, Lebanon, N. H.
F. M. Webster, January 22, 1906, Tyngsboro, Mass.
W. J. Morse, May 24, 1906, Burlington, Vt.
W. B. Hall, March 19, 1906, Wakeman, Ohio.
G. I. Reeves, January 22, 1907, Pine City, Minn.
J. B. Weems, June 14, 1906, Crewe, Va.
J. L. Phillips, April 5, 1905, Blacksburg, Va.
W. J. Phillips, November 8, 1904, Bonsack, Va.
J. L. Phillips, August 30, 1905, Fort Wayne, Ind.
G. I. Reeves, December 9, 1904, Lincoln, Nebr.
L. Bruner, March 1, 1905, Lincoln, Nebr.
W. J. Phillips, October 21, 1906, Richmond, Ind.
G. I. Reeves, November 1, 1906, Marquette, Mich.
G. I. Reeves, November 1, 1906, Chatham, Mich.
G. I. Reeves, November 3, 1906, Sault Ste. Marie, Mich.
G. I. Reeves, November 24, 1906, Corning, N. Y.
C. V. Piper, September, 1907, Turkestan, Asia.
C. N. Ainslie, October, 1907, Jefferson, Ohio.

CLASSIFICATION AND DESCRIPTION

Tetrastichus bruchophagi Gahan belongs to the hymenopterous superfamily Chalcidoidea, family Eulophidae, and subfamily Tetrastichinae. It was described as a new species by Mr. A. B. Gahan,[1] of the Bureau of Entomology, from type specimens reared by the writer from *B. funebris* infesting alfalfa seeds at Corcoran, Cal. Mr. Gahan's description follows.

Tetrastichus bruchophagi. Female.—Length 1.8 mm. Antennal pedicel and the three funicle joints subequal in length, the club about as long as the two last funicle joints combined; head not wider than the thorax; malar space long, equaling or nearly equaling the height of the eyes; whole head finely lineolated with a few round punctures on the cheeks, prothorax finely punctured, mesoscutum and scutellum finely lineolated, the parapsidal grooves deep and broad, the median line of the mesoscutum distinct but fine; two longitudinal grooves on the mesoscutellum very distinct, the distance between them not equal to half the length of the scutellum; metanotum about half as long as the propodeum and faintly sculptured, propodeum with faint subreticulate sculpture similar to that of the metanotum, the median carina distinct; abdomen conic ovate, as long as head and thorax.

Color dark blue-green; antennæ very dark brownish, the apex of scape below, and underside of pedicel paler; all coxæ, trochanters, and femoræ greenish black; apices of all femoræ, all tibiæ, and the tarsi, except apical joint, pale yellow.

Male.—Similar to the female except for secondary sexual characters.

Type locality.—Corcoran, Cal.

[1] Gahan, A. B. New Hymenoptera from North America. *In* Proc. U. S. Nat. Mus., v. 46, p. 431–443, pl. 39. 1914.

STAGES OF HOST SHOWING PARASITISM

Tetrastichus bruchophagi is parasitic upon the larval stage of its host, *B. funebris*. This parasite attacks the half-grown or fully developed host larva, and after attaching itself punctures the host larval skin, feeding upon the body contents. It is normally an external parasite but in several instances has been found to be completely inclosed within the host larva.

One hundred and eleven larvæ of *T. bruchophagi* under observation showed 106 of these parasite larvæ as externally parasitic and 5 as internally parasitic upon their respective host larvæ. All of the specimens counted in this test were reared to the adult stage for determination. This species has not been observed to be parasitic upon the pupal stage of its host.

HIBERNATION

This species hibernates within infested seeds of alfalfa in which it has attacked its host, and spends the winter in the larval stage. Larvæ becoming fully developed as early as August and September frequently go into hibernation. In a few instances this species was observed to enter the pupal stage late in the fall and to pass through the winter in this stage. Most of the pupæ and undeveloped larvæ are killed by the first severe frost in the fall.

APPEARANCE IN THE FIELDS

Tetrastichus bruchophagi is probably the first of the parasites of *B. funebris* to appear in the fields in early spring. A comparative study of larvæ of this species, together with other parasites of *B. funebris*, shows that the larvæ of *T. bruchophagi* are among the first to transform to the pupal stage and emerge as adults with the approach of warm spring weather. The adults may be seen among the first blossoms of alfalfa and are active over the first developing seed pods.

Table I shows the emergence of adults of *Tetrastichus bruchophagi*, reared from larvæ which had hibernated through the winter; and Table II the emergence of adults of the summer generations as they appeared in the laboratory, from alfalfa seed infested with *B. funebris* and taken from the fields.

TABLE I.—*Emergence of adults of Tetrastichus bruchophagi from hibernated larvæ*

Period of emergence.	Male.	Female.
Mar. 1–15	.....	.....
Mar. 16–31	3	1
Apr. 1–15	6	1
Apr. 16–30	10	6
May 1–15	7	17
May 16–31	21	31
June 1–15	5	52
June 16–30	18	109
July 1–15	4	48
July 16–31	1	2
Aug. 1–15	1	.....
Total	76	267
Percentage of sexes	22.15	77.8

TABLE II.—*Emergence of adults of the summer generations of Tetrastichus bruchophag as they appeared in the laboratory, from alfalfa seed infested with B. funebris and taken from the fields*

Period of emergence.	Male.	Female.
July 1–15	1	20
July 16–31	47	417
Aug. 1–15	71	243
Aug. 16–31	3	72
Sept. 1–15[1]	.....	.....
Sept. 16–30[1]	.....	.....
Oct. 1–15[1]	.....	.....
Oct. 16–31	8	12
Nov. 1–15	4	6
Nov. 16–30	.....	.....
Total	134	770
Percentage of sexes	14.82	85.17

[1] Adults emerged freely, but counts of sexual forms were not made.

OVIPOSITION

This minute parasite searches over the green alfalfa seed pods for those containing seeds infested by *B. funebris*. When a suitable place is found for oviposition, the female suddenly lowers the tip of her abdomen to the surface of the seed pod and forces the ovipositor into the seed pod and infested seed, placing an egg upon the larva of its host. The time required for oviposition varies from about 20 seconds to 1 minute.

LARVA

DEVELOPMENT.—The larva of *T. bruchophagi* upon hatching from the egg finds itself upon the host. In the course of one or two days it takes a more or less permanent position and begins to feed. A very rapid growth follows the first feeding of this parasite. In the course of its develop-

ment indistinct molting is apparent, and fragments of the cast-off larval skin may be noticed peeling off of the developing larva. The fully developed larva has frequently been found to be almost inclosed within the old larval skin. In other cases this cast-off skin has been observed to be worked back over the body of the larva.

DORMANT PERIOD.—After the larva has become full grown, further development toward the pupal stage depends largely upon external climatic conditions to which the seed is subjected. Complete drying of the seed containing this parasite delays pupation, and, in fact, may produce a dormant period to the larval stage of indefinite duration, or until both humidity and temperature are favorable for further development. This dormant period frequently begins in early summer and carries the species to the following year before further development takes place. Material kept in the laboratory showed adults of this species emerging almost two years after the seeds containing the parasite larvæ had been collected in the field.

DESCRIPTION.—The larva (Pl. 78, *A*) varies in color from clear white to smoky white, and some of them have been found to be almost cream colored. They average 1.3 mm. in length and 0.66 mm. in diameter. The general shape is elliptical when at rest. At times the larva takes on a pear-shaped form. It has a small head and 13 body segments. The first three body segments back of the head are slightly wider than the others. The body is free from pubescence and smooth, very lightly segmented. The mandibles are very small and almost invisible.

LENGTH OF LARVAL STAGE.—The length of the larval stage depends greatly upon the duration of the dormant period into which it enters, but under the most favorable conditions this period does not require more than about 10 days and pupation follows soon after the larva is fully developed.

PREPUPAL STAGE.—Before entering the pupal stage, a prepupal period of about 48 hours' duration occurs. During this time the formation of the pupa within the larval skin takes place and the larva loses its normal shape.

PUPA

PUPATION.—After the pupa (Pl. 78, *B*) has formed within the larval skin, the latter breaks open along the dorso-anterior margin and is worked back until it is free from the body of the pupa.

DESCRIPTION.—The pupæ measured averaged 1.2 mm. in length and 0.7 mm. in width. At first the pupa is white, but later the eyes take on a brown color and the body becomes black. The body of the pupa is rather straight, with head bent slightly forward. The appendages are folded close to the body and the pupa is covered by a thin pupal skin. Before changing to the adult stage the pupa becomes almost black, with a blue-green metallic luster.

LENGTH OF PUPAL STAGE.—The pupal stage seems to vary greatly, depending upon temperature conditions of the season in which pupation occurs. Larvæ which had hibernated through the winter went into pupation as early as March, in California, and some of the hibernating larvæ under less favorable conditions did not enter pupation until two or three months later.

Fifty-two males reared from hibernating larvæ showed an average of 17.7 days in the pupal stage. Fifty-six females reared from hibernating larvæ showed an average of 19.7 days in the pupal stage. In midsummer the pupal stage, as observed in the laboratory, showed an average of 6.5 days. The shortest pupal period observed was 6 days, and the longest pupal period, not considering hibernation in that stage, was 35 days.

ADULT

EMERGENCE.—The adult (Pl. 78, *C*) upon emerging from the pupal skin finds itself completely inclosed within the walls of the alfalfa seed, which has been hollowed out by its host, *B. funebris*. It gnaws a small hole through the seed wall and through the seed pod, making its escape.

RELATIVE PROPORTION OF SEXES.—In connection with the rearing of adults of this species from different localities throughout California and western Arizona, it was found that both sexes were well represented, but that the females were considerably more abundant than the males. A total of 1,249 adults examined showed 210 to be males and 1,039 to be females, or a ratio of 1 to 4.94.

SEASONAL HISTORY

There are from two to four generations of this species in a single season, where conditions are favorable, as is the case in the alfalfa seed fields of central California. Under the less favorable, hot and dry desert conditions the species frequently has only a single generation in a season. The development from egg to adult of each generation is completed within the unbroken alfalfa seed infested by its host.

IMPORTANCE AS A PARASITE

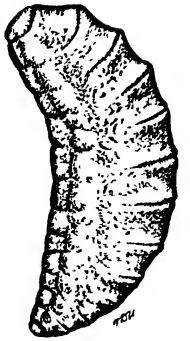
Tetrastichus bruchophagi is a parasite of considerable importance in controlling *Bruchophagus funebris* in the alfalfa seed-growing districts of central California, where it apparently destroyed about 52 per cent of the chalcis-fly larvæ infesting alfalfa seeds in 1913.

In the alfalfa seed fields of the Yuma, Ariz., district this parasite is apparently of little importance at the present time. Observations show that it destroys only about 1.5 per cent of the chalcis-fly larvæ in that seed district.

PLATE 78

Tetrastichus bruchophagi

A.—Larva.
B.—Pupa.
C.—Adult.



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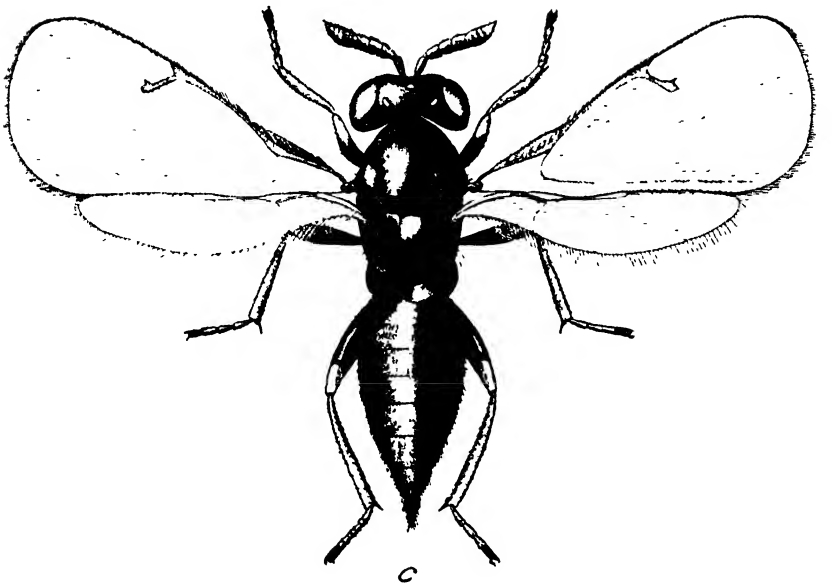

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JOURNAL OF AGRICULTURAL RESEARCH

VOL. VIII WASHINGTON, D. C., FEBRUARY 19, 1917 NO. 8

ARSENICAL INJURY THROUGH THE BARK OF FRUIT TREES

By DEANE B. SWINGLE, *Botanist and Bacteriologist*, and H. E. MORRIS, *Assistant Botanist and Bacteriologist, Montana Agricultural Experiment Station*

INTRODUCTION

The economic aspects of our interest in the effects of arsenical compounds upon plant life have come to center largely about three conditions that have developed as a result of recent commercial practices, (1) the injury to vegetation by arsenic from smelters, (2) the injury to foliage and fruit by arsenical insecticides, and (3) the possible injury to fruit trees through the bark of the crowns and roots by the corrosive action of arsenicals that have accumulated there as a result of spraying.

In the year 1909 we began at this Station an extensive investigation to determine, so far as possible, all the effects of arsenical compounds upon vegetation. The scope of this work is far-reaching, including the effects of practically all known compounds of arsenic on many different species throughout the plant kingdom. The studies have so far been largely physiological, but it is evident that much chemical work also will be required to solve some of the problems involved.

This paper takes up only one phase of the investigation, but as our work on that is practically complete and it is of immediate interest to orchardists, entomologists, and pathologists, the results are being published in advance of the rest, on which much more work will be required. A preliminary report (10),[1] embodying our first year's work on this subject, was published in 1911.

After fruit trees have been sprayed with improperly prepared arsenical insecticides, it has occasionally been noted that the bark of the twigs has been more or less injured, but in such cases the damage to leaves and fruit has been so severe as to attract much greater attention. This is but natural, for, as the fruit is more resistant to this injury than the leaves, and as the bark is even more resistant than the fruit, it is evident that any arsenical mixture that could be recommended as doing only moderate damage to the leaves would have no visible effect whatever upon the bark.

[1] Reference is made by number to "Literature cited," p. 317–318.

HISTORICAL REVIEW

In 1907, Whipple (11), then Field Horticulturist of the Colorado Experiment Station, in discussing certain rootrots and crownrots that came under his observation, suggested that the more destructive one which "seems to work exclusively in the Ben Davis and Gano * * * may be due to arsenic collecting about the crown of the tree and killing the bark." As he did not carry on extended experiments to prove arsenic to be the cause, and as he suggests that there is some evidence that the disease is infectious, the affected trees being often in groups, this brief note did not arouse much interest.

The next year, however, Headden (5), Chemist of the Colorado Experiment Station, sounded a note of warning to the effect that we would do well to use moderation in applying arsenical compounds to fruit trees for the control of the codling moth and other insect pests and that it would be better, indeed, if these insecticides could be replaced by others not containing arsenic. He offered evidence to show that in Colorado at least much damage was being done by the arsenicals collecting about the crowns of the trees and killing the bark there and eventually the trees themselves, and that, too, in considerable numbers. If we sift from his bulletin the evidence upon which he bases his belief that arsenic is responsible for the killing of the trees in question, we may briefly state it as follows:

1. The trees are dying from injury to the bark on the crowns and bases of the roots, and the cause has not been previously found, though a search for parasites has been made by other members of the Colorado Experiment Station staff.

2. The trees have been sprayed with arsenicals, sometimes in excessive amounts, and these have accumulated in the soil, especially close around the crowns.

3. It is a fact quite generally accepted that solutions of arsenicals are poisonous to plants.

4. Pure water will, to some extent, bring lead arsenate and other so-called insoluble compounds of arsenic into solution, and if the alkali salts frequently found in Colorado soils are added to the water the arsenicals are dissolved to a much greater extent.

5. Arsenic in considerable quantity was found in the soil under the trees and in the injured trees themselves.

6. A tree known to have been badly injured by sodium arsenite poured into an irrigation ditch near it presented symptoms similar in some important respects to the many dying trees under discussion.

In a later bulletin (6) he strengthens some of these points of evidence, but emphasizes the fact that, while the alkali salts make the arsenicals more soluble and are doubtless of importance in some orchards, they are not at all necessary to bring a portion of the arsenic into solution, and in

some orchards where they are not present to any considerable extent in the soil the trees show corroded crowns and an abundance of arsenic in the wood. In the same bulletin he also cites (p. 19) an observation that is of especial interest in this connection and is as follows:

> Dr. S. M. Bradbury, Horticultural Inspector of Mesa County for eight years, makes the following statement, which is used with his permission. "Bands had been used in a certain orchard and on adopting the lime, sal soda and arsenic spray these bands were left on the trees, some of them for two or three years. Some of these trees died and on examining the bands we found that the bark beneath the bands had been entirely eaten up. The lime and arsenic had collected under the bands and destroyed the bark."

While Headden does not claim that this evidence is proof positive that arsenical compounds are responsible for the death of the trees, he is evidently thoroughly convinced that such is the case. His line of argument presents some points of weakness. He does not prove that trees that are uninjured, or not obviously injured, may not contain as much arsenic as the injured ones. He does not prove that the arsenic was taken through the corroded crowns or that, though poisonous if absorbed through the feeding roots, it can attack the cork-protected bark. He does not prove that arsenic in small quantities is injurious to fruit trees nor does he establish the point of concentration above which it becomes injurious. He does not establish points of difference between the injury in question and that found in other States on trees planted in virgin soil to which no arsenic has ever been applied for any purpose and where, therefore, arsenic can not be a factor in causing the injury. While some vain search for parasitic fungi and bacteria has been made at the Colorado station, their absence is very difficult to prove conclusively, as testified to by the many unsuccessful early attempts to discover the crowngall organism.

At least two investigators dissent from Headden's conclusions. Ball, of the Utah Experiment Station, would attribute the cause directly to alkali and seepage water, and Grossenbacher, formerly of the New York Experiment Station, shows some points of similarity to a form of crown-rot prevalent in New York and elsewhere which he attributes to winter injury. These discussions will be reviewed later in this paper (see p. 316).

This very suggestive work of Headden's, while not accepted by all as conclusive, has made a strong impression upon many horticulturists, entomologists, and plant pathologists over the country and has pointed out the necessity for a study of the physiological aspects of the questions. It is highly important to determine beyond all dispute whether or not trees are injured at the crowns by arsenical spray mixtures and, if so, the conditions upon which this injury is dependent. We have, therefore, treated numerous branches of various sizes, trunks, crowns, and roots with different arsenical compounds, including those used as insecticides, to study the injury to bark and wood if any should be produced.

EXPERIMENTAL METHODS

We have endeavored, among other things, to reproduce as nearly as possible the conditions obtaining about the crowns of the trees as a result of spraying, especially with reference to the presence of the arsenicals in immediate contact with the bark, and the moisture necessary for bringing them into solution. When crowns were to be treated, this was accomplished by carefully removing the earth without injuring the bark, applying the arsenical, and replacing the earth. The natural precipitation and the regular orchard irrigation were depended upon to furnish the necessary moisture. This method, while simulating closely the conditions brought about by orchard spraying, is obviously cumbersome. It is slow of application, frequent examination to note the course of the injury is difficult, and as each tree could be used for only a single treatment a good-sized orchard would have been required to furnish trees for all the tests we have made, the cost of which would have been prohibitive. We therefore made considerable use of trunks and branches.

When the trunks were treated, this was done by a method shown us at the Utah Experiment Station by Dr. E. D. Ball, to whom we are indebted for the suggestion. A funnel of white rubber sheeting was constructed about the trunk and sealed to it with tree tanglefoot (Pl. 79). Inside this, the arsenical was placed, distilled water was added, and the whole covered with a hood of rubber sheeting sealed to the trunk above and loosely overhanging the funnel. This served to keep out rain water and permitted free access of air. This method is open to most of the objections mentioned above and, furthermore, the rough, shaggy bark of the trunk makes it very difficult to keep the funnels from leaking. If one attempts to smooth this up by scraping there is considerable danger of making unnatural breaks in the corky bark. It is not unlikely that Dr. Ball used a more satisfactory wax than tree tanglefoot. As a matter of fact we used this method in only nine cases—those reported in Table VIII.

The great majority of the tests recorded in this paper were made on branches of various sizes, and the chemical was held in contact with them by means of a bandage of absorbent cotton protected by a wrapping of surgeon's gauze, the whole being wet with distilled water. As the orchard had never been sprayed, it seemed desirable at the outset to prevent the escape of any of the arsenic from the bandage to the soil so that any injury that might occur could not possibly be attributed to absorption through the feeding roots. This end was attained in the early experiments by selecting branches that showed a decided sag at some point and placing the bandage at the lowest part. A metal box was hung close under the bandage to catch any drip produced by rains. Later we tried wrapping the bandages in a dental rubber dam to prevent the drip, and omitting the boxes (Pl. 80). In repeated tests the two methods gave results almost identical, so the boxes were discarded as they were more cumbersome,

required more frequent watering, and were applicable only to branches having the right kind of sag, which in time became difficult to find.

In those bandages that were not covered with rubber the conditions of moisture and aeration were closely similar to those often found about the crowns a few inches beneath the surface of the ground. They were wet with distilled water every day, but owing to our atmospheric conditions, that favor rapid evaporation, they were, as a rule, nearly or quite dry an hour or more before the daily watering. Those that were wrapped in rubber were wet continuously and the aeration was not so good, but many comparative tests gave results almost identical by the two methods, or as nearly identical as the duplicates were by either method alone. We were, therefore, led to believe that excessive moisture and imperfect aeration did not materially influence the injury, which had usually begun in a few days and was complete within two or three weeks from the time of treatment and probably before the bark would make any abnormal growth due to lack of air or excessive moisture.

In the few cases where roots a little distance from the crown were treated it was by the same methods as described for the branches. Root treatments required so much labor, however, that only enough were made to assure us that the susceptibility of roots is not greatly different from that of branches. This work was further complicated by the difficulty of finding roots of suitable size that did not bear rootlets which, when broken off by digging, presented unnatural openings for the entrance of arsenical solutions.

This work was all carried on in the college orchard at Bozeman, where some branches could be injured and removed without serious damage to the remainder and where, by good fortune for this work, a few rows of trees were to be removed entirely and were available for the most severe treatment of crowns, trunks, and large branches.

The tests were made almost entirely upon apple trees, though pear and plum were tried in a few cases. Where the latter were used, they are specifically mentioned; otherwise, the results described in the following pages apply entirely to the apple.

ARSENICAL COMPOUNDS USED

In carrying on work of this kind much difficulty is encountered in determining the exact composition of arsenical compounds regularly put upon the market or even in getting made to order all the different compounds wanted, free from mixture with other arsenicals. There is a great tendency on the part of insecticide companies to prepare mixtures, especially of the diplumbic and triplumbic lead arsenates. We were fortunate, however, in securing, on special order from the J. T. Baker Chemical Co., of Phillipsburg, N. J., most of the more important arsenicals apparently quite pure.

There exists much confusion of names for the ortho lead arsenates. There seem to be only two that are commonly manufactured as insecticides. These are the diplumbic and the triplumbic lead arsenates. The diplumbic is also called "monoplumbic," "acid," and even "pyro." The triplumbic is variously termed "neutral," "normal," "basic," and "ortho" lead arsenate.

Following is a list of the chemicals used, with the percentage of arsenic that each contained as determined by the chemistry department of this Station. The arsenic was calculated on a moisture-free basis.

	Per cent
Arsenic acid, Merck	51.10
(Merck & Co., New York and Darmstadt.)	
Ammonium arsenate, Merck	45.20
Ammonium arsenite, Merck	68.45
Arsenic trisulphid (yellow), Baker	59.10
(J. T. Baker Chem. Co., Phillipsburg, N. J.)	
Arsenic trisulphid (yellow), Merck	26.12
Arsenic trioxid, Baker	73.10
Arsenic trioxid, Merck	75.40
Calcium arsenite, Baker	51.20
Calcium arsenite, Merck	44.78
Copper aceto arsenite (Paris green), Ansbacher	42.42
(Ansbacher Insecticide Co., Inc., New York.)	
Copper aceto arsenite (Paris green), Baker	42.85
Iron arsenate, Merck (a mixture of ferrous and ferric)	38.40
Lead arsenate, diplumbic, Baker	21.31
Lead arsenate, triplumbic, Baker	16.88
Lead arsenate, mixture, Grasselli	19.21
(Grasselli Chemical Co., Cleveland, Ohio.)	
Lead arsenate, diplumbic, Grasselli	21.13
Lead arsenate, triplumbic, Grasselli	14.60
Lead arsenate, "technical," mixture, Merck	19.25
Lead arsenate, mixture, Swift	20.19
(Merrimac Chemical Co., Boston, Mass.)	
Lead arsenate, diplumbic, Swift	
Potassium arsenate, Merck	41.32
Potassium arsenite, Merck	49.70
Sodium arsenate, Merck	24.75
Sodium arsenite, Merck	58.15
Zinc arsenite, mixture, Baker	53.00
Zinc arsenite, meta, Baker	52.75
Zinc arsenite, ortho, Baker	31.90
Zinc arsenite, pyro, Baker	41.33
Zinc arsenite, Merck	35.10
Zinc arsenite, ortho, California Spray Co.	32.14
(California Spray Co., Watsonville, Cal.)	
Zinc arsenite, "Ortho 40," California Spray Co	24.38

NATURAL PROTECTION OF THE BARK

It has long been known that the outer portion of the bark of many trees is made up of a layer of cork varying in thickness from few to many cells. On apple trees (*Malus sylvestris*) this forms a well-developed

coating on all parts except the leaves and the youngest stems and roots. This layer of cork is well formed on the young shoots when they go into their first winter and persists thereafter. It forms a layer that is smooth and for the most part fairly even in thickness until the limbs and trunks reach a considerable size. As the limb enlarges inside this layer, new cork cells are added from within. The older cork cells on the outside slough off to some extent, but, as a rule, no definite breaks are found until the natural formation of rough bark begins. This takes place when the trunk or the limb is from 2 to 6 inches in diameter, depending upon variety and environmental conditions.

The well-known roughened condition of the bark is brought about by rifts or splits forming in the outer corky layer and the tissues lying just beneath it (Pl. 81, *A*). These often begin at the intersection of branches, in crotches, and about old wounds and other irregularities, and gradually extend over the entire trunk and the larger limbs (Pl. 81, *B*). While these cracks form more or less gradually and cork is produced later at their inner extremities, there is often a period when the living cells of the bark are wholly unprotected in places by the cork cells. Thus, it happens that while it might seem at first thought that the heavy, rough bark of trunks and crowns is a thicker and better protection than is found upon the smaller branches, such in reality is not the case; for while the cork of this rough bark is in some places very thick, in others it is quite broken through. Table I records a series of observations made in the late fall of 1910 upon the thickness of the cork on branches of different ages. Further examinations made in July, 1915, indicate that there is very little difference between the dormant and active periods.

TABLE I.—*Number of cells in periderm of smooth bark and in the furrows of rough bark of apple trees*

Year's growth	Character of bark.	Diameter.	Cells in cork layer.	Character of bark.	Diameter.	Cells in cork layer.	Character of bark.	Diameter.	Cells in cork layer.
		Inches.			*Inches.*			*Inches.*	
1910	Smooth[a]	1/4	3–5	Smooth[c]	1/16	1–3	Smooth[e]	1/16	2–5
1909	do	1/4	2–5	do	1/16	4–6	do	1/16	2–5
1908	do	1/16	3–5	do	3/8	3–7	do	3/4	2–5
1907	do	3/8	4–6	do	1/16	3–7	do	3/8	3–7
1906	do	3/8	4–8	do	1/2	4–7	do	3/8	2–7
1905	do	5/8	4–9	do	3/4	3–7	do	5/8	4–8
1904	do	1/4	4–8	do	7/8	2–8	do	1/8	3–7
1903				do	1 1/8	5–7			
1902	Smooth[a]	1/8	3–8	do	1 3/8	1–8			
1900	do	1 1/8	4–10						
1899	do	1 1/4	4–9						
1896	do	2 1/4	4–9						
	do	4	4–many	Smooth[d]	4	4–many	Smooth[e]	4	3–many
	Rough[b]	6	2–many	Rough[d]	6	4–many	Rough[f]	6	6–many
	do	8	5–many				do[g]	8	3–many
	do	12	1–many						

[a] Okabena. [b] Transcendent crab. [c] Hibernal. [d] Liebe. [e] Wealthy. [f] Oldenburg. [g] Orange.

OPENINGS THROUGH THE CORK LAYER

Even on the smooth bark, where the layer of cork is for the most part fairly even, the protection offered is not perfect. At the time the periderm is forming, the lenticels are forming also. On apple bark these are quite numerous and of varying sizes (Pl. 82, *A*, *B*). While they have not been studied with such thoroughness as those on some other woody plants, it is well established that they have numerous, though rather small, intercellular spaces and permit free access of air for several years. As the stem grows in diameter, they enlarge very much and on some varieties they divide into groups. From their appearance under the microscope (Pl. 83, *A*, *B*) we would judge that some of them at least remain functional for several years, sometimes until nearly the time when the bark becomes furrowed and roughened. While the lenticels are known to admit air, the extent to which they will admit liquids can be determined only by experiment. On the smooth bark the lenticels are the only natural openings after the disappearance of the stomata, but artificial openings can easily be made and do frequently occur as a result of hail, insects, and various orcharding operations. The rough bark, while less easily broken by external agencies, is frequently cracked by the growth within, as described above. The roots also, after reaching a diameter of ¼ to ½ inch, have lenticels in varying sizes and numbers, which for the purposes of this investigation are not essentially different from those on the aerial portions (Pl. 82, *C*).

ARSENICAL INJURY THROUGH THE SMOOTH BARK

When arsenical compounds of almost any kind are applied to the branches by the methods described above, if the quantity is sufficient, more or less injury follows. In many cases the natural protection of the cork and the penetrability of the lenticels and wounds are clearly shown.

CHARACTER OF INJURY

Roughly speaking, we may say that the various compounds of arsenic produce the same symptoms of injury, varying only in degree.—that is, a given amount of injury by one compound is very much like the same amount produced by another. Calcium-arsenite injury in differing severity will therefore be described; and let it suffice for a general description of arsenical injury through the smooth bark, keeping in mind only that with us some of the milder compounds like ortho zinc arsenite and iron arsenate have never produced the severest injury.

When the bark is unwounded and the injury is only slight, it is not visible to the eye for several days, usually a week or more, depending somewhat upon the temperature, and perhaps other conditions. Then it may be noticed that close around certain lenticels is a small indefinite zone which is slightly darker in color on the surface than the surround-

ing bark. This forms a tiny spot, with the lenticel in the center (Pl. 83, *C*). If the affected tissues are cut into with a knife, they are found to be darkest near the surface; but the color grades imperceptibly into that of the healthy bark a millimeter or two below the surface. Each day now for about a week or two the spot becomes a little darker externally and internally and increases a little in size. It then becomes sharp in outline, as a rule, and quite definitely sunken. Two or three weeks after treatment, if made during the growing season, most of the spots cease to grow in size. They are then very dark brown, almost black, externally and much sunken, and a crack begins to form at the boundary between the injured tissues and the healthy ones. These spots vary in size from one to several millimeters and often anastomose into irregular dark patches. As a rule, the spots are larger on the under side of a horizontal branch than on the upper. The difference, however, is somewhat less noticeable if the bandages are covered with a rubber dam and thereby kept evenly wet. In a spot 5 mm. in diameter the tissues are killed and colored a very dark brown to the cambium and the youngest wood is sometimes affected also. In a spot 10 mm. in diameter the bark is discolored to a depth of one to several millimeters and there is considerable injury to the underlying wood. The affected bark is not friable at this time, but tough and leathery. Not all the lenticels are affected, as a rule; sometimes only a few and sometimes many, from which we may judge that some of them are closed at times.

If the action of the chemical is more severe, many lenticel spots are usually found anastomosed and the dead patch thus formed may girdle the limb under the bandage. From this dead patch one or more streaks may run up and down the limb. These vary in length and diameter, and their size may be used to indicate the severity of the attack. In extreme cases they are several feet long and kill some of the side branches. These streaks, like the spots, are at first indefinite and later become nearly black and sunken. The wood under the dead bark is also discolored, varying from a light coffee-brown to nearly black. This discoloration may invade only the wood next to the cambium, but often it extends to the pith.

When a wound is made through the outer corky layer just before the chemical is applied, the tendency to form streaks is much more marked than when the absorption is through lenticels. There will appear around the wound more or less discoloration and in mild cases the injury stops here. Sometimes near-by lenticels will show spots also. There is a strong tendency, however, to form a streak running up and down from the wound (Pl. 84). As a rule, it extends farther upward than downward; but the reverse is sometimes true. Sometimes the whole branch above the bandage is killed, and only a streak runs down. Sometimes the streaks run a spiral course, and if there are two wounds on opposite sides of the branch the two streaks from them keep on opposite sides and

run a parallel course even though both are spiral. As a rule, the streaks are continuous, but occasionally they are intermittent, disappearing from view and reappearing higher up. In all such cases examined, however, the streak in the wood was continuous, the span of sound tissue being only in the bark. These streaks may show almost every proportional variation in length and width. There is a strong tendency for the streaks that run down to be terminated by intersection with a larger branch, but there are frequent exceptions.

The leaves on some part or all of the affected branch may also show more or less injury. The first symptoms may be apparent as early as the first or second day after treatment. The leaves at first lose their normal luster and look dull and a little lighter green. This condition may affect the entire leaf, but often only spots and irregular areas. In bad cases there is considerable wilting. Soon the spots so affected turn brown and become dry and crisp. It is a notable fact that the leaves so affected are practically always directly touched by a discolored streak or area. A twig touched by such a streak may have every leaf killed, while on the opposite side of the limb, even if it is only a half inch in diameter, a twig will have leaves with no visible trace of injury.

In studying the symptoms of injury through the smooth bark in many different cases, one is impressed by the great uniformity of certain general features and the great variety of differences in minor details. The uniform and constant symptom is localized discoloration of bark and wood, beginning as an indefinite discoloration and ending after a period of a few weeks as a sharply defined, dark, sunken area. If the absorption of arsenic is slight, the discolored region does not extend much farther longitudinally than transversely; but if the absorption is considerable, as through a wound, a long and relatively narrow streak is produced. Beyond these general features there is little uniformity, the relative length and breadth of the streaks, their relative length above and below the point of absorption, and their branching at intersections, with side branches presenting an endless variety of detail.

A feature of this work that has given us constant trouble is the frequent failure of duplicate and triplicate tests to check with each other. When two or three applications were made under conditions apparently identical in every respect, the resulting injury was not always the same in extent and character. In the majority of cases the results were nearly enough alike for practical purposes (Table VI); but all too frequently duplicate and triplicate tests gave widely different results, as may be seen, for example, in Tables IV and V. These differences were most marked in limbs that had been wounded just before treatment and gave much less trouble in those that were unwounded. As a result of this erratic behavior, most of the tests were repeated over and over again for confirmation; and the reader is warned against drawing hasty and definite conclusions

from a comparison of any two treatments. It is possible that a thorough anatomical and histological study of branches that showed wide differences under identical treatment might have revealed the explanation, but the importance of the question did not seem to warrant the expenditure of time.

One of the most striking facts connected with this type of injury is the self-limitation of the streaks. At the end of two weeks they have practically always run their course, unless the treatment was given in late fall or winter. For a week or two longer the margins continue to grow sharper and the dead bark becomes more sunken, but almost never is there any material lengthening or broadening. The explanation of this early limitation is not clear to us. At first it seemed possible that the chemicals used contained some soluble impurity which became entirely absorbed during the first week, after which there was little injury; but this idea has been wholly abandoned. We have used chemicals made up with especial care as to purity, and we have washed them in many changes of distilled water, but the results in this respect were the same (see p. 302). Also, we get this same limited course from bandages kept saturated with the soluble arsenicals, such as sodium arsenite and arsenic acid. We have established quite conclusively that this discolored wood and bark conducts the solutions very poorly; this probably has much to do with the phenomenon. However, it must be remembered that liquids pass rapidly up the xylem ducts and some would be expected to reach the leaves at the tip of the branch very quickly before any visible change takes place. That some arsenic does actually reach distant parts that continue to appear healthy has been established by chemical tests made by the Station chemist. It seems quite probable that by the action of the tissues through which the chemicals pass some of the arsenic is withdrawn from solution, enough so that what passes on is too weak to cause the complete death of the cells or a noticeable discoloration of the ducts.

To determine further the relation of the absorption of solutions through wounds to the killing of tissues by the solutions absorbed, the following experiment was conducted: Three wounded limbs were each treated with 10 gm. of calcium arsenite, three others with 10 gm. of calcium arsenite and 1 gm. of methylene blue, and three others with 1 gm. of methylene blue alone. No injury was produced by the stain alone, and the injury by the arsenical alone was about like that with the arsenical plus the stain. When examined a month after treatment, it was found that where the stain was used alone it made relatively narrow streaks up and down from the wound. These streaks were not visible externally and affected only the youngest of the inner bark and extended into the wood for a depth of one to three annual rings. They were of an intense blue color near the wound

and became paler and narrower until they disappeared. Where the stain was used with the arsenite, it was the same in character as where used alone, but was always much narrower than the dead streak surrounding it and usually shorter. Also, several side limbs were more or less injured by the arsenical, but contained no visible trace of the blue. In no case did the blue extend into living wood or bark. These results would suggest that the solutions absorbed by the wounds are not carried very far beyond the dead portions.

INJURY AS AFFECTED BY CHARACTER OF WOUNDS

Some questions arise as to the relative importance of wounds of different character. Are small abrasions sufficient to make injury possible? Does a wound just through the periderm admit the arsenic as freely as one extending to the cambium or into the sapwood? When do wounds like those made by pruning become sufficiently closed to keep out the solution?

From a study of Table II it is evident that even a very slight rupture of the periderm is sufficient to make possible severe injury to the limb if the arsenical is applied continuously in a moist state (10, p. 86). So far as these few tests go, we may infer, however, that the chemical is absorbed less rapidly if the wound is superficial than if it extends to the cambium. If, however, the slower injury, like that through the superficial wound in No. 3, results in a girdling of the limb, it is just as surely fatal to the portion above as though absorption were more rapid. In comparing the injury through wounds made by cutting off twigs and branches with that through wounds made by cutting out a small piece of bark to the cambium, we find that sometimes the injury is worse through the former and sometimes through the latter. On an average, there is not a great deal of difference.

TABLE II.—*Results of wounding and treating branches of Early Strawberry apple on August 21, 1911, with 5 gm. of Baker's copper accto arsenite, to show the relative injury through wounds of different size and character*

No.	Diameter of treated branch.	Wound under bandage.	Extent and character of injury.
	Inch.		
1	¾	Branch ¾ inch cut off.	Streak half around branch runs down from wound 1 foot and up 3 feet to the tip.
2	¾	One-fourth inch square of bark removed to cambium.	Streak half around branch runs down 7 inches from wound and up 4 feet nearly to the tip.
3	¾	One-fourth inch square of outer cork removed.	Streak half around branch runs down from wound 2 inches and up 3 inches.
4	¾	Transverse cut through bark to cambium on one side.	Girdled under bandage, except for a small strip on side opposite wound.
5	¾	Radial cut 1 inch long through bark to cambium.	Streak from wound runs down 7 inches and up 20 inches.
6	¾	Five fine needle pricks through bark.	Entirely girdled under bandage. Streak runs down 5 inches to main limb and up nearly to tip, 2½ feet.
7	¾	Old pruning wound..	Injury extends 1 inch up and 1 inch down from wound with little at the sides. A few lenticel spots.
8	¾	Unwounded.....	Numerous lenticel spots ¼ inch or less in diameter.
9	¾	.....do.....	Numerous lenticel spots ¼ inch or less in diameter. Two small spots around latent buds.

Table III shows quite conclusively that the injury through wounds of a few months or older is much less than through freshly made wounds. This has been tested at different times on branches other than those listed in this table, and it has been our general observation that while there is sometimes more or less darkening of the wood through wounds made several months or even two or three years before treatment, there is rarely any serious injury to bark or leaves. A few exceptions have been observed.

TABLE III.—*Results of treating branches of apple on July 15, 1912, with 25 gm. of Baker's calcium arsenite, to show the relative injury through wounds of different age and character*

No.	Diameter of treated branch.	Wounds under bandage.	Extent and character of injury.
	Inches.		
1	1	Branch 1 inch diameter cut off 4 years ago.	A few lenticel spots.
2	1⅛	Branch cut off 4 years ago. Partly healed.	Slight discoloration on under side of branch under bandage.
3	1⅛	Branch 1¼ inches diameter cut off 3 years ago and completely healed over.	Branch under bandage almost girdled by anastomosing lenticel spots. No special injury around wound.
4	2¼	Branch 1½ inches diameter cut off 3 years ago. Partly healed.	Practically no injury.
5	1⅛	Bark peeled off ⅛ by 1⅛ inches 3 years ago.	Indefinite streaking to partly killed side branch 2½ feet above bandage. A few lenticel spots.
6	1⅛	Branch 1 inch diameter cut off 2 years ago.	No visible injury.
7	2¼	Branch ¾ inch diameter cut off in winter of 1912.	A few small lenticel spots under bandage.
8	1½	Branch cut off in late winter of 1912.	Nearly girdled under bandage. No special injury around wound.
9	2⅛	Branch ¾ inch diameter cut off 2 months ago.	No visible injury.
10	¾	Two twigs cut off 2 days before treatment.	Branch killed above and under bandage. Sunken streak runs from each wound to tip.
11	1⅛	Small branch cut off and notch cut to cambium 2 days before treatment	Branch killed above and under bandage. Sunken streak runs to tip, 4 feet.
12	1	Twig and small branch cut off just before treatment.	Branch killed above and under bandage. Sunken streak runs to tip, 4½ feet.
13	¾	Twig cut off and notch cut to cambium just before treatment.	Branch killed above and under bandage Streak runs down 1 foot, injuring 3 side branches.

PROTECTION OF WOUNDS BY PAINTING

It was suggested in our preliminary report (10) that white-lead paint applied to wounds, even though they were freshly made and the arsenical was applied before the paint was dry, would offer some measure of protection. This suggestion was based on very few trials, which left it in need of further investigation. Paint was therefore applied to wounds of various sizes and ages and these were afterwards treated with copper aceto arsenite or, in a few cases, calcium arsenite. More than 50 painted wounds were so treated. In some cases the chemical was applied as soon as the paint was put on and in others the paint was allowed to dry for different lengths of time. The wounds were made either by cutting off a small twig or by cutting a notch through the bark to the cambium.

Seven wounded limbs were painted with white-lead paint and immediately treated with copper aceto arsenite. There was considerable

injury in every case. The least was a streak running up 11 inches and down 3 inches. In the worst injury the entire branch above the bandage was killed, and a dead streak ran down 10 inches. Between these two extremes there were all gradations. In comparison with the injury through unpainted wounds, it was clear that the paint had in some cases given a partial protection, but in others little or none.

A few wounds were left unpainted in the center to see whether the arsenical entering through the wood at that point would work outward and cause an injury to the bark. The failure of the paint to protect when applied to the entire cut surface leaves the result doubtful in these cases.

Twenty-one other wounds of a similar nature were painted with white-lead paint as soon as made, and, after an interval for drying, 10 gm. of copper aceto arsenite was applied to each. On four of these the paint was allowed to dry for 6 hours before treatment, on six others it was allowed to dry for 7 hours, and on the remaining eleven 24 hours. The work was done in dry, sunny weather; and even in 6 hours the paint showed considerable evidence of drying. In 24 hours it seemed quite dry, though not very hard. In general, the paint under these conditions reduced the amount of injury very materially as compared with unpainted wounds similarly treated, and yet in nearly every case streaks several inches long were produced and in four cases the entire branch above the bandage was killed. In three of these four cases the paint had dried for 7 hours and in the other 24 hours.

Eleven branches varying in size from 1½ to 3⅛ inches were cut off several inches from the large limbs on which they grew, so that long stubs were left. These stubs were painted on the cut ends and for about 2 inches back on the uninjured bark. Some were given one coat and some two, with an interval of 24 hours for drying after each coat. The end of each stub was treated with 25 gm. of Baker's calcium arsenite, the bandage being applied in the form of a cap and covered with a dental rubber dam. In some cases, especially where two coats of paint had been applied, the action of the chemical was considerably lessened, but in the majority, including some with two coats of paint, the injury was excessive, running down the stub and into other parts of the tree. In no case was the protection complete.

Nine other stubs were similarly treated with spar varnish, but this offered no better protection than the paint.

It should be noted in this connection that in these large stubs the pressure of sap was so great as to form blisters under the paint and varnish; and these often broke open, which would easily account for the injury. This condition, however, did not occur on the wounds on smaller limbs.

If it were a matter of great practical or scientific importance, it is possible that a covering could be found that would give better protection than either white-lead paint or varnish.

RELATIVE INJURY TO BRANCHES OF DIFFERENT SIZES

As it was difficult to find enough branches of exactly the same size to carry on this entire investigation and as there is some purely scientific interest in knowing whether smaller branches are more or less susceptible than larger ones, especially through the lenticels, a series of tests was made on limbs and branches of as widely varying sizes as could be found without including any having rough bark.

Twelve unwounded branches were treated with 10 gm. each of Baker's calcium arsenite. These varied in size from ¼ inch to 2½ inches. Of the three that were only ¼ inch in diameter, two were killed above the bandage, and the other was nearly girdled under the bandage but uninjured above and below. The rest all showed anastomosing lenticel spots. On the branches less than 1 inch in diameter the enlargement of these spots had resulted in a complete girdling, and in some cases in streaks several inches long. On the larger branches, of which there were six, the spots were a little more distinct and about alike on all, with no streak above or below the bandage.

If, then, the branches are unwounded, we find that in those between ½ inch and 1 inch in diameter there is no consistent difference in susceptibility dependent upon size. There is sometimes found a considerable variation on smaller-sized branches even on the same tree; but, between the limits just named, size seems to be an unimportant factor. Unless otherwise specified, the branches described in the tables of this paper were from ⅝ to ⅞ inch in diameter. Branches as small as ¼ inch in diameter appear to be worse injured. Branches more than an inch in diameter show less streaking than the smaller ones, though the lenticels absorb the arsenicals almost as freely. The thickness of the bark seems to be a factor here. Except for the fact that the branches are eventually killed by girdling under the bandage through anastomosing lenticel spots, the injury does not extend as far from the bandages as in the smaller branches.

Twelve other branches varying in size from ¼ inch to 2½ inches were wounded by cutting off a small twig or by cutting a small notch to the cambium. Each was then treated with 10 gm. of Baker's calcium arsenite. The injury to these limbs was very severe. All less than 1 inch in diameter were killed above the bandage and there were long streaks below. Those more than 1 inch across (except one) were badly injured above the bandage but not entirely killed, and the streaks below were much shorter than on the smaller branches. The one exception just mentioned, which was 1⅛ inches in diameter, was the worst injured of any. It was killed above the bandage and a wide dead streak ran down 4 feet to the trunk and out a large side branch 6 feet to its tip.

It would seem from this that, when branches have been wounded so as to expose both phloem and xylem ducts, there is no consistent difference

in amount of injury between the smaller branches and the larger until a diameter of 1 inch is reached. The portion above the point of treatment is more often entirely killed in the smaller branches than in the larger, but this is because there is less tissue to kill above the point of treatment. The largest two in this series showed somewhat less injury than the rest, but one much larger than the average was the worst injured of the 12.

It is, of course, possible that had a milder chemical been applied than that used in this experiment greater differences due to size of branch would have developed; but with several opportunities during the course of the investigation to make isolated observations along this line we saw no reason to believe that size of branch is an important factor in determining the extent of injury, except that lenticel injury through a thick bark extended less rapidly than through a thin bark. This statement applies only to limbs with a smooth bark.

RELATIVE INJURY BY DIFFERENT ARSENICAL COMPOUNDS

That equal amounts of different chemical compounds injure plant tissues in different degrees is to be expected. In the first place, many of these compounds are readily soluble in water, and others are only slightly so and are often called "insoluble." All the arsenical insecticides used for spraying plants are of the latter character. As the solubles are more readily absorbed than the so-called insolubles when abundant moisture is present, it seems best to compare the solubles with each other and the insolubles with each other. In making such comparisons it would be possible either to use equal weights of the different chemicals or such quantities as to give equal amounts of arsenic. Either basis would be logical for its own purposes, and the former—that is, equal weights of the chemicals—was chosen for this investigation.

Considering, first, the soluble arsenicals, we have compared them on both wounded and unwounded limbs. These chemicals were dissolved in water in proportions between 1 to 1,000 and 1 to 1,000,000 and the bandages were saturated with the solution, evaporation being practically prevented by wrapping with a dental rubber dam.

Twelve unwounded branches from ½ to ¾ inch in diameter were treated with soluble compounds as follows: Two each with arsenic acid, ammonium arsenate, potassium arsenate, potassium arsenite, sodium arsenate, and sodium arsenite in the strengths of 1 to 1,000 and 1 to 5,000 by weight. Arsenic acid in each strength caused a small lenticel spot. Potassium arsenate, 1 to 1,000, caused a few small lenticel spots. Sodium arsenite 1 to 1,000 caused a few small lenticel spots and a spot ⅜ inch in diameter about a latent bud. The others caused no injury.

Thirty-six other branches received two wounds each, either by cutting off twigs or by cutting a small notch through the bark to the cambium.

The same compounds were used as on the unwounded branches just described. The strengths varied between 1 to 10,000 and 1 to 1,000,000. In a strength of 1 to 1,000,000 none of the chemicals caused injury. In a strength of 1 to 100,000 there was no injury other than a slight discoloration of the margin of the wounds, except in the case of potassium arsenate, which in one of the duplicate treatments killed the bark for ⅛ to ⅝ inch around the wound. In a strength of 1 to 50,000 practically no injury was caused by potassium arsenate, potassium arsenite, or sodium arsenate. The bark was killed for $\frac{1}{16}$ to ⅛ inch about the wound by arsenic acid and for ⅛ to ¼ inch by sodium arsenite. Ammonium arsenate caused a spot ½ by 1 inch about the wound. When used in a strength of 1 to 10,000, there was some injury from every chemical, and in most cases definite streaks ran up and down from the wounds. The average length of these streaks from the top of the one running up from the wound to the bottom of the one running down was as follows: Arsenic acid, 16 inches; ammonium arsenate, 2 inches; potassium arsenate, 4 inches; potassium arsenite, ¾ inch; sodium arsenate, ¾ inch; sodium arsenite, 1¼ inches. As these figures are averages for four wounds in each case, they probably give a fair idea of the toxicity of the soluble arsenical compounds.

It may be seen that when the limbs are not wounded they will withstand much stronger solutions than if the bark is ruptured. A strength of 1 to 10,000 materially injured wounded limbs; but 10 times that strength, or 1 to 1,000, caused very little injury to unwounded limbs, and in most cases none at all.

Turning now to a consideration of the relative injuriousness of the so-called insoluble arsenicals, we find much wider differences than in the solubles. Here we have to deal with two factors: The solubility of the chemicals under the conditions of the experiments and the toxicity of the part in solution.

To get a comparison of the effects of different insoluble arsenicals on unwounded branches, the following were applied in 60-gm. quantities:

Arsenic trioxid, Baker.
Arsenic trioxid, Merck.
Arsenic trisulphid, Baker.
Arsenic trisulphid, Merck.
Calcium arsenite, Baker.
Calcium arsenite, Merck.
Copper aceto arsenite, Ansbacher.
Copper aceto arsenite, Baker.
Lead arsenate, diplumbic, Baker.
Lead arsenate, diplumbic, Swift.
Lead arsenate, triplumbic, Baker.
Lead arsenate, triplumbic, Grasselli.
Lead arsenate, technical, Merck.
Zinc arsenite, ortho, Baker.
Zinc arsenite, ortho, California Spray Co.

Two unwounded branches were treated with each chemical. The severest injury was caused by arsenic trioxid, arsenic trisulphid, calcium arsenite, and Baker's triplumbic lead arsenate. These all produced streaks extending up and down from the bandages for several

inches. Under the bandage the branch was girdled by at least one of the duplicate treatments. Zinc arsenite (California Spray Co.) caused no injury whatsoever. The amount of injury caused by the other chemicals was intermediate, consisting of lenticel spots that were anastomosing more or less.

Twelve other unwounded branches were treated in duplicate with the following chemicals, 10 gm. each:

Calcium arsenite, Baker.	Zinc arsenite, ortho, Baker.
Iron arsenate, Merck.	Zinc arsenite, pyro, Baker.
Copper aceto arsenite, Baker.	Zinc arsenite, meta, Baker.

In this series calcium arsenite entirely killed the branch above the bandage. Streaks ran down from the bandages 16 and 20 inches, respectively. Copper aceto arsenite and the three zinc arsenites caused only lenticel spots, which in some cases were anastomosing. Iron arsenate caused no injury.

It is to be noted that in this last series 10-gm. quantities of calcium arsenite and of copper aceto arsenite caused more injury than did 60 gm. in the series just described. The 60-gm. series was begun on July 30, 1911, and the 10-gm. series on June 17, 1912, both in midsummer.

Had the injury in these two series been approximately equal, it would not have been especially remarkable, as we have found that, as a rule, the injury from these slightly soluble arsenicals is by no means proportional to the amounts used. Erratic results like these have made us very careful about drawing conclusions concerning the exact amounts required to produce injury, and have prompted us to make comparisons only between treatments given on the same date under conditions as nearly alike as possible.

We may at least conclude that even unwounded limbs may be severely injured or ultimately killed as a result of girdling by some of the chemicals having only slight solubility. It appears, however, that tests on wounded limbs, where the exposure of living tissues to the chemicals is assured, are somewhat more suitable for comparing the toxicity of the chemicals than those on unwounded limbs where the corky bark limits their free access.

Tables IV and V record the results of work specifically intended to determine the relative injuriousness of the more common insoluble arsenical compounds when applied to branches that are wounded. A close study of these tables shows some points of interest. One of the most striking facts is that similar tests, made under conditions as nearly alike as possible, do not always yield the same result. Briefly stated, duplicate tests do not always check with each other, though in most cases they do so fairly well. In other words, there are factors that we can not control, and do not even know, that sometimes influence to a marked degree the

extent of injury. This introduces an experimental error that must not be overlooked. Iron arsenate and one brand of zinc arsenite (California Spray Co.) have been conspicuous in all the tests by the small amount of injury which they produce. Other brands of zinc arsenite have proved more injurious. It is obviously unsafe to make general statements about "ortho zinc arsenite" without naming the brand. Indeed, we have no assurance that different lots of any brand will always be harmless. The same may be said of the lead arsenates. Even those manufactured as pure diplumbic or triplumbic show some differences. Two arsenicals have quite regularly given severe injury: Calcium arsenite and arsenic trioxid. Arsenic trisulphid has usually given less injury than these and copper aceto arsenite still less, though more, as a rule, than the ortho zinc arsenites and most lead arsenates.

TABLE IV.—*Results of wounding and treating branches of Hibernal apple on June 23, 1911, with different arsenicals to show their relative injuriousness. Those with even numbers were covered with a dental rubber dam and those with odd numbers were not* (*see p. 286*)

No.	Chemical.	Extent of injury			
		60 gm. of chemical.		10 gm. of chemical.	
		Above bandage.	Below bandage.	Above bandage.	Below bandage.
			Inches		*Inches.*
1	Arsenic trioxid, Baker	Entire branch	44	Entire branch	[a] 31+
2	do	do	27	do	14
3	Arsenic trioxid, Merck	do	29	do	29
4	do	do	41	do	25
5	Arsenic trisulphid, Baker	do	29	do	16
6	do	do	33	do	13
7	Arsenic trisulphid, Merck	11 inches	4	do	34
8	do	Entire branch	18	do	8
9	Calcium arsenite, Baker	do.	22	do	[b] 9+
10	do	do	39	do	18
11	Calcium arsenite, Merck	do	[b] 14+	do	[a] 10+
12	do	do	[b] 16+	do	13
13	Lead arsenate, diplumbic, Baker	do	40	do	15
14	do	do	29	do	6
15	Lead arsenate, diplumbic, Swift	Streak to tip	2	1 inch	1
16	do	Entire branch	5	18 inches	1
17	Lead arsenate, triplumbic, Baker	do	2	36 inches	2
18	do	do	5	Entire branch	6
19	Lead arsenate, triplumbic, Grasselli	do	20	do	5
20	do	Streak to tip	3	1 inches	3
21	Lead arsenate, technical, Merck	Entire branch.	7	Entire branch.	1
22	do	do	11	Streak to tip	5
23	Copper aceto arsenite, Baker	do	18	12 inches	10
24	do	do	12	Entire branch	2
25	Copper aceto arsenite, Ansbacher	do.	54	do	9
26	do	do	12	Streak to tip	3
27	Zinc arsenite, ortho, California Spray Co	None	None	None	None.
28	do	do	do.	do	Do.
29	Zinc arsenite, mixture, Baker	Entire branch	18	Entire branch.	10
30	do	do.	11	Streak to tip	5
31	Zinc arsenite, mixture, Merck	do	13	8 inches	1
32	do	do	19	Entire branch.	1

[a] No. 1 (10 gm.) and 11 (10 gm.) grew from the same main limb and the streaks ran together on it.
[b] No. 9 (10 gm.), 11 (10 gm. and 60 gm.), and 12 (60 gm.) grew from the same main limb, and the streaks from all three bandages ran together upon it, making it impossible to say how much of the common streak was caused by any one of them.

TABLE V.—*Results of wounding and treating branches of apple on June 17, 1912, with different arsenicals, 10 gm. each, to show their relative injuriousness*

No.	Chemical.	Extent of injury.	
		Above bandage.	Below bandage.
			Inches.
1	Calcium arsenite, Baker	Entire branch.	19+
2	do	do	20
3	Iron arsenate, Merck	½ inch	½
4	do	6 inches	2
5	Copper aceto arsenite, Baker	36 inches	12
6	do	Entire branch.	5
7	Zinc arsenite, ortho, Baker	9 inches	8
8	do	30 inches	2
9	Zinc arsenite, pyro, Baker	10 inches	2
10	do	Streak to tip.	2
11	Zinc arsenite, meta, Baker	Entire branch.	12
12	do	do	32

[a] Nineteen inches below the bandage this branch joined a main limb bearing a dead streak from another bandage, and the two ran together.

It is interesting to compare in Table IV the relative amounts of injury from 60 gm. and 10 gm. of the insoluble arsenicals under identical conditions. While there is somewhat greater injury, as a rule, from the larger amount, it is not at all proportional to the weights used.

Table IV also shows that there was no consistent difference between the injuries under bandages covered by a rubber dam and those not so covered, but kept moist by frequent watering. On the whole, they compare about as well as the duplicates in the other tables.

In general, the injury is much worse on wounded limbs, and nearly all the so-called insoluble arsenicals are capable of doing much damage to the tree if they gain access to the living tissues.

EFFECTS OF WASHING THE ARSENICALS

The question has arisen with much force whether the injuries produced in the course of this investigation were due to a portion of the arsenical going into solution and exerting a toxic action or to an impurity of a more or less soluble nature. This is indeed an important matter, for an assumption here without knowledge of the facts may lead one into a serious and fundamental error. In our efforts to settle this question we have, in the first place, obtained most of our chemicals from reliable companies. In some cases we have had special lots manufactured for our use, with the distinct understanding that regardless of cost they must be free from all soluble arsenical impurities. All of the arsenicals received from the Baker Chemical Co. (see p. 287) were of this character. To test further the possibility of soluble impurities we washed one lot of each of the more important chemicals with many changes of distilled water. These were then tested in comparison with others from the same containers but unwashed.

The results show that the washing may have slightly decreased the injury in some cases. This was not uniformly true, for with arsenic trioxid and ortho zinc arsenite the washed chemical caused fully as much injury as the unwashed, and with copper aceto arsenite and triplumbic lead arsenate the results were nearly alike. In fact diplumbic lead arsenate was the only chemical that showed a striking improvement as a result of washing. This is probably due, as suggested by Dr. Headden,[1] to a change in the composition of the lead arsenate from the diplumbic to the triplumbic as a result of washing. A close comparison of the results is fruitless, owing in part to the rather large experimental error of the methods here used. The essential fact is that both washed and unwashed chemicals of the purest brands obtainable do cause severe injury.

EFFECTS OF ADDING LIME TO THE ARSENICALS

It has frequently been recommended that lime be added to arsenical insecticides, especially copper aceto arsenite, to lessen the possibility of injury when sprayed on foliage. To determine whether this would materially lessen the injury under the conditions of these experiments—namely, when the arsenicals are applied to the wounded bark—1 gm. of partly air-slaked lime [2] was mixed with the arsenical contained within each bandage. The following arsenicals, 10 gm. each, were used, two with lime and two without:

Calcium arsenite, Baker.
Copper aceto arsenite, Baker.
Lead arsenate, diplumbic, Baker.
Lead arsenate, diplumbic, Grasselli.
Lead arsenate, triplumbic, Baker.
Lead arsenate, triplumbic, Grasselli.
Zinc arsenite, ortho, Baker.
Zinc arsenite, "Ortho 40," California Spray Co.
Lime only.

A comparison of the injury with and without lime shows little evidence that lime lessens the injury from any of the chemicals tested. It appeared at first glance to lessen the injury from Grasselli's triplumbic lead arsenate and Baker's ortho zinc arsenite, but as it failed to do so with Baker's triplumbic lead arsenate and "Ortho 40" zinc arsenite, we are led to believe that this apparently beneficial effect is largely a matter of coincidence. We are strengthened in this belief by the fact that several duplicate tests in this series differ so much from each other. The lime alone caused no injury.

DIFFERENCES IN VARIETIES OF APPLE, PEAR, AND PLUM

Field observations have led to the belief among horticulturists that there is a difference in varieties of apple with reference to the resistance of their foliage to injury by arsenical spray mixtures. The Ben Davis

[1] This suggestion was offered verbally by Dr. Headden in 1910.

[2] Quicklime was allowed to air-slake until it had taken a powdered form, but was still strongly alkaline to litmus paper.

is reported to be easily injured, and the Gano shares somewhat this reputation, not so much apparently from actual observation as from inference because of the close relationship of the two varieties. Headden (5, 7) refers to this special susceptibility of the Ben Davis and the Gano and notes that under some conditions at least these two varieties are most frequently victims of crown injury. So far as the spray injury is concerned, this special weakness of the Ben Davis and the Gano may be due either to poorer protection of the leaf cuticle (or possibly to some difference in the stomata) or to a greater susceptibility of the living interior portion. Likewise, if the injury described by Headden is caused by arsenic which has accumulated about the crown, the more frequent injury which he observed in these varieties may have been due to a structural difference in the protective outer bark or to a difference in the resistance of the inner tissues.

More than 100 tests were made on wounded and unwounded branches for direct comparisons, under practically identical conditions, of the relative susceptibility of varieties. The following were included in these tests: Alexander, Ben Davis, Gano, Hibernal, Okabena, Wealthy, and Yellow Transparent apples; Hyslop and Transcendent crab apples; Flemish Beauty pear, and De Soto and Moldarka plums.

In addition to the tests planned for this specific purpose, many others made for different purposes gave additional opportunity for comparisons of varietal susceptibility. A fairly representative series is shown in Table VI.

TABLE VI.—*Results of wounding (twig cut off or notch cut to cambium) and treating different varieties of apple and plum on June 14, 1915, with 10 gm. of Baker's copper aceto arsenite, to compare susceptibility of varieties*

No.	Diameter of treated branch.	Variety.	Extent and character of injury.
	Inch.		
1	5/8	Ben Davis apple. . .	Dead above bandage except for narrow strip that runs up 14 inches. Streak runs down 2 inches. Several lenticel spots.
2	5/8	.....do.....	Streak nearly around branch runs up 5 feet to tip. Narrow streak runs down 3 inches. Nearly girdled under bandage.
3	5/8	... .do.....	Dead above bandage except for narrow strip that runs up 1 foot. Streak half around branch runs down 10 inches.
4	5/8	Gano apple	Dead above bandage except for narrow strip that runs up 10 inches. Streak runs down 6 inches.
5	5/8	.....do.....	Streak half around branch runs up 4 feet to tip and down 6 inches. Large spot at latent bud.
6	3/4	.....do.....	Streak half around branch runs up 3 feet to tip and down 10 inches to main limb and on down this 6 inches.
7	5/8	Hibernal apple.....	Dead above bandage except for narrow strip that runs up 2 feet. Streak runs down 2 inches.
8	1/2	.....do.....	Dead above bandage. Streak half around runs down 6 inches to main limb.
9	5/8	.....do.....	Wide streak runs up 22 inches. No injury below bandage. Several lenticel spots.
10	5/8	Okabena apple.....	Streak two-thirds around branch runs up 3 feet to tip. Wide streak runs down 6 inches. Three lenticel spots.
11	5/8	.....do.....	Dead above bandage except for narrow strip that runs up 16 inches. Wide streak runs down 2 feet to main limb. Several lenticel spots.
12	3/8	.....do.....	Streak two-thirds around branch runs up 4 feet to tip. No injury below bandage. Several lenticel spots.
13	5/8	Oldenburg apple.....	Streak half around limb runs up 9 inches to side branch and out this 14 inches. Wide streak runs down 7 inches. Several lenticel spots.
14	3/8	.....do.....	Dead above bandage except for narrow strip that runs up 15 inches. Streak half around runs down 7 inches.

TABLE VI.—*Results of wounding (twig cut off or notch cut to cambium) and treating different varieties of apple and plum on June 14, 1915, with 10 gm. of Baker's copper aceto arsenite, to compare susceptibility of varieties*—Continued

No.	Diameter of treated branch.	Variety.	Extent and character of injury.
15	5/8	Oldenburg apple.....	Dead above bandage except for narrow strip that runs up 5 inches. Two streaks run down 5 inches.
16	5/8	Wealthy apple.....	Streak half around branch runs up 3 feet to tip and down 4 inches. Several lenticel spots.
17	5/8	...do.....	Do.
18	5/8	....do.....	Streak half around branch runs up 3½ feet to tip. Narrow streak runs down 3 inches. Several lenticel spots.
19	5/8	Yellow Transparent apple.	Streak half around branch runs up 5 feet to tip. Narrow streak runs down 5 inches. One lenticel spot.
20	5/8	.....do.....	Streak half around branch runs up 2 feet. Wide streak runs down 7 inches. Several lenticel spots.
21	3/4	.....do.....	Wide streak runs up 2½ feet. Narrow streak runs down 5 inches. Several lenticel spots.
22	1/2	Hyslop crab.....	Dead above bandage except for narrow strip that runs up 6 inches. Wide streak runs down 8 inches.
23	5/8	.....do.....	Streak two-thirds around branch runs up 2½ feet to tip. Wide streak runs down 5 inches. Many lenticel spots
24	5/8	.....do.....	Streak half around branch runs up 18 inches and down 3 feet. Several lenticel spots.
25	1/2	Transcendent crab...	Dead above bandage. Wide streak runs down 8 inches.
26	1/2	.....do...	Streak three-fourths around branch runs up 4 feet to tip. Streak runs down 3 inches.
27	1/2	.....do.....	Streak half around branch runs up 5 feet to tip. No injury below bandage.
28	1/2	De Soto plum.....	Streak half around branch runs up 3 feet to tip Streak runs down 8 inches.
29	3/4	.....do.....	Streak half around branch runs up 2½ feet to tip and down 2 inches.
30	1/2	.....do.....	Streak half around branch runs up 3½ feet to tip. Wide streak runs down 5 inches to main limb and on down this 1 foot.

Throughout our work we find little evidence of any important difference in the susceptibility of varieties of apple to arsenical injury when the chemical is admitted through wounds. If conclusions were drawn from this table alone, it might be inferred that the Wealthy is somewhat more resistant than the others; but this is not borne out by other tests. It may be said further of the tests reported in this table that the triplicates check with each other fully as well as the majority made in this investigation. The few tests made of pear and plum lead to the belief that they are not very different from apple under these conditions—namely, when the arsenical is applied to wounded branches ½ to ¾ inch in diameter.

In the tests on unwounded branches to compare the susceptibility of varieties, 10 gm. of copper aceto arsenite were used in most cases. This produced lenticel spots but no streaking on every treated branch of every variety of apple tested. The number and size of these spots seemed to vary about as much between the duplicates or triplicates on the same variety as between varieties, and we got no evidence of special susceptibility or resistance in any variety of apple. In the Flemish Beauty pear there was a much greater tendency for the lenticel spots to enlarge and anastomose, and in the Moldarka plum there was a discoloration of all the outer bark which rarely reached the cambium. Possibly, if the bandages had been kept on for a very long time, a gradual extension of the injury might have taken place in which some varietal difference would have been apparent.

From these repeated experiments, therefore, it seems safe to conclude that when arsenicals are applied by this method to either wounded or unwounded limbs they injure the different varieties of apple about equally. Certainly neither the Ben Davis nor the Gano is especially susceptible under these conditions. However, as the governing factor seems to be the outer protective covering, and as this covering is very different on the smooth-barked limbs and on the rough-barked crowns extensive tests on the latter might yield different results. No Ben Davis or Gano trees were available for crown treatment, however, and many might be required to make conclusions free from experimental error. Without these two varieties such a test would miss the point of greatest interest.

EFFECTS OF SEASON UPON INJURY

As this investigation was carried on during several months of spring, summer, and fall, it is of some importance to know whether the season or stage of growth influences the extent or character of injury. To determine this, a considerable number of tests was made during every month from March to October, inclusive. From the specific treatments made for this purpose and others that have contributed to our information on the relation of season to injury, we conclude that when the trees are frozen the injury is very restricted, being confined wholly to the exposed tissues of the wounds. When the trees are more or less active, even before the leaves come out in the spring and after they drop in the fall, the injury will readily extend a considerable distance from the point of treatment. Throughout this active period the extent of injury from a given treatment is about the same, but it runs its course a little more rapidly just after the leaves come out and more slowly about the time they are dropping in the fall.

INJURY THROUGH THE ROUGH BARK OF LIMBS, TRUNKS, AND CROWNS

Casual observation might lead one to believe that the heavy, rough bark on the trunks of trees is a better protection against all kinds of harmful agencies than the thin, smooth bark of the small branches. That it does offer a greater protection against mechanical injuries is undisputed, and it is also true that the thicker, projecting portions of the rough bark are highly protective against the absorption of arsenical compounds when added to the unwounded surface. Between these thick ridges however, are the furrows in which the corky layer is more or less broken. As the tree expands in diameter new rifts are formed or the old ones are reopened. Microscopic study confirms what close macroscopic observation suggests, namely, that the rough bark has vulnerable points of a character not found in the periderm of the branches above.

It has often been observed that when a tree is thoroughly drenched with a spray mixture a considerable quantity of this runs down the trunk

to the ground. Some lodges in cracks and other irregularities of the bark on the trunk; but this is not likely to cause injury, as it soon dries out. Around the crown, however, it accumulates to a much greater extent and is often kept moist by the soil for long periods of time. As the crown of the tree is covered by the rough or furrowed bark which theoretically offers only partial protection, it is highly important to determine its vulnerability by actual test.

INJURY TO LARGE LIMBS WITH ROUGH BARK

A few large limbs were found having longitudinal cracks in the bark of the kind just described. These limbs were treated with copper aceto arsenite or calcium arsenite under bandages such as were used for most of this investigation. Care was used to make no artificial wounds. The results are shown in Table VII.

TABLE VII.—*Results of treating large unwounded limbs of apple showing the first cracks of rough bark formation, on July 13, 1912, to show injury through rough bark*

No.	Chemical (50 gm.).	Diameter of treated limb.	Natural cracks in bark, apparently recent.	Extent and character of injury.
		Inches.		
1	Copper aceto arsenite, Baker.	3¼	One short, deep, longitudinal crack 5 inches long.	Limb completely girdled under bandage and for about 2 inches above.
2	....do	3½	Irregular longitudinal crack about 3 inches long.	Limb girdled under bandage and for about 3 inches above and below.
3	....do.....	2¾	Deep longitudinal crack 6 inches long.	Limb girdled under bandage and about 3 inches above and below. Streak runs up 10 inches.
4	Calcium arsenite, Baker.	2¼	Longitudinal crack 7 inches long Lower portion recently formed	Limb girdled under bandage and about 7 inches above and 3 inches below.
5	.. do....	4	Six longitudinal cracks on under side of limb. Not all recent.	Limb girdled under bandage. Wide streak runs up 2½ feet and down to ground 2½ feet.
6	..do	1¼	Irregular cracking on one side	Limb girdled under bandage Streak runs up 2 feet and down 6 inches.

It will be seen from this table that in every case the limb was severely injured. The absorption was rather slow and there was little tendency to form the dead, sunken streaks up and down the limbs so characteristic of injury through wounds. In this respect there was a close similarity to the crown injury under controversy in Colorado and elsewhere. Lenticel injury was out of the question in these cases, as the lenticels had become nonfunctional and indeed had practically disappeared from the larger limbs.

INJURY TO TRUNKS

A few trunks also were treated early in the course of this investigation. The method here was to apply the chemicals in funnels about the trunks as described on page 286.

That the bark on the trunk fails to protect the tree from arsenical injury is shown by Table VIII. The absorption was evidently slow,

judging from the absence of symptoms in the foliage for several weeks after the trunks were treated. Owing to the dark color and firm texture, the heavy bark does not show externally the injury that is taking place until long after the tissues are dead; and it seemed undesirable to disturb the experiments by cutting into the bark until there was other evidence that injury had taken place.

TABLE VIII.—*Results of treating the unwounded trunks of apple trees with different chemicals applied on July 12, 1912, to show injury through rough bark*

No.	Chemical.	Injury 2½ months after treatment.[a]	Injury 8½ months after treatment.
1	Sodium arsenite, Merck, 10 gm.	Bark dead for 8 to 12 inches above funnel. Nearly half the leaves killed. Bark and wood of branches not discolored.	Tree dead throughout. Bark dark and friable below and under funnels, especially around the crowns where covered by earth.
2	.....do.....	Bark above funnel nearly all sound. Streak runs up 2 feet. About half the leaves killed and many of the remainder spotted.	Do.
3	Arsenic trioxid, Baker, 500 gm.	Two streaks run up trunk about 1 foot. No leaves killed or spotted. About half the fruit softening and streaked throughout with brown. No untreated trees have fruit in this condition.	Trunk girdled under funnel. Streak runs up 6 inches. Bark dead below funnel to the roots. Rather dark colored and friable.
4	.....do.....	No dead bark above funnel. Otherwise like No. 3.	Trunk girdled under funnel. Streak runs up 2 feet. Otherwise like No. 3.
5	Copper aceto arsenite, Baker, 500 gm.	No dead bark above funnel. Fruit softened and streaked like No. 3.	Trunk girdled under funnel and irregular patch of dead bark extends several inches above. Bark below funnel killed on one side of trunk to roots. Below ground it is dark reddish brown and friable.
6	.....do.....	No visible injury to bark, leaves, or fruit.	Trunk girdled under funnel. Wide streak on one side extends from funnel nearly to ground.
7	Lead arsenate, triplumbic, Baker, 500 gm.	Bark sound above bandage. Fruit softened and discolored like No. 3.	Funnels left for later notes (see first paragraph following this table.
8	.. do	No visible injury to bark, leaves or fruit.	Do.
9	Water only (control)..	No visible injury...	No injury of any kind.

[a] When these first notes were taken, the funnels were not removed and the injury below the funnels could not be traced externally. When the last notes were taken the funnels were removed and the trees cut up.

It will be noted from the table that No. 7 and 8, the two trees treated with lead arsenate, were not disturbed at the time the notes were taken, 8½ months after treatment. It was desired to ascertain whether or not the injury would continue, these two being the only trees not girdled and therefore the only ones we could hope would survive. One of these trees, No. 7, was removed from the orchard through a misunderstanding before final notes were taken. The other, No. 8, showed no injury externally on October 28, 1914, 2¼ years after treatment. On removing the funnel and cutting into the bark on that date, however, a dead spot was found under the bandage from which a streak ran down to the roots.

It is worthy of note that in these cases where the absorption was slow the visible injury was not always continuous as where the absorption was rapid through a wound. Most of the bark and sapwood between the funnel and the discolored leaves in No. 1 and 2, 2½ months after treatment, was normal in appearance, and in No. 3, 4, and 7 most of the bark and sapwood was not discolored 8½ months after treatment, although the fruit had shown injury the previous September.

INJURY TO CROWNS

In treating the crowns of trees it was deemed advisable to place the chemicals very much as they accumulate as a result of spray mixtures running down the trunks. To accomplish this, the earth was removed around the crowns to a depth of a few inches, care being taken not to injure the bark in the slightest degree, except on certain trees that were purposely wounded. The arsenicals were then distributed in these shallow trenches against the bark and the earth replaced. Except in applying the chemicals, no water was added during the course of the experiments, the natural moisture of the soil being depended upon to keep some arsenic in solution at least a portion of the time. Fresh applications were given in three successive years, the last one being twice the strength of the first two.

The experiments recorded in Table IX show that even small quantities of arsenic trisulphid or copper aceto arsenite are capable of causing injury to the unwounded crowns. This suggests that in time the injury might even girdle the trees. That calcium arsenite would have caused a more serious injury in the same length of time seems highly probable, judging from a comparison with the other chemicals when applied to branches. We must not, however, overlook the possibility that the soil solution about the crowns influenced the relative injuriousness of the chemicals.

TABLE IX.—*Results of treating the crowns of apple trees three successive years with different chemicals, to determine character of injury*

No	Treatment	Diameter of trunk	Wounds at crown	Injury, Mar. 25, 1913
		Inches		
1	Arsenic trioxid, Merck; 7 gm July 19, 1910, and Aug. 22, 1911, 14 gm Aug 8, 1912.	4½	Unwounded	Uninjured.
2	do	4¾	Two strips of bark ¾ by 1½ inches removed just below ground.	Narrow streak runs up 3 feet. Roots uninjured.
3	Arsenic trisulphid, Merck; 15 gm July 19, 1910, and Aug 22, 1911; 30 gm Aug. 8, 1912.	4¾	Unwounded	Several dead spots about 1 inch in diameter extend into bark, but none reach the cambium
4	do.	4¼	Same as No. 2..	Wide streak runs up 3 feet, but does not extend down onto roots. Several dead spots about 1 inch in diameter extend into bark. The tissue is very dark and friable. Two spots extend through bark into wood.
5	Lead arsenate, technical, Merck; 60 gm. July 19, 1910, and Aug. 22, 1911, 120 gm Aug 8, 1912.	2	Unwounded ...	Apparently uninjured Aug. 8, 1912. Removed before final notes were taken.
6	..do...	4½	Water sprouts cut from crown	Do.
7	Copper aceto arsenite, Ansbacher; 15 gm. July 19, 1910, and Aug. 22, 1911; 30 gm. Aug. 8, 1912.	3¼	Unwounded.....	Several small dead spots on crown, but none reached the cambium.
8	.....do.....	3	Water sprouts cut from crown.	Wide dead streak runs up 4 feet. This starts from a large dead patch on the crown. Roots uninjured.
9	Zinc arsenite, ortho, California Spray Co.; 15 gm. July 19, 1910, and Aug. 22, 1911; 30 gm. Aug. 8, 1912.	4¼	Unwounded.....	No injury.
10	.....do.....	4	Same as No. 2..	Tree broken down by storm.

When trees are wounded at the crown, as by the cutting off of water sprouts, which is a fairly common orchard practice, the danger of arsenical injury is greatly increased, as shown by a comparison of the wounded and unwounded trees in this table.

To determine whether a more rapid injury could be produced by larger applications of more injurious chemicals, the tests were made that are recorded in Table X. A study of this table leaves no doubt that the crown of an apple tree is very poorly protected against the absorption of arsenicals. Three days after treatment nearly every tree showed some symptoms in the foliage and at the end of a week every tree was badly injured. In a few weeks every crown was completely girdled.

TABLE X.—*Results of treating unwounded crowns of apple trees on July 19, 1912, with 500 gm. of Baker's calcium arsenite, to determine the extent and character of injury*

No.	Diameter of trunk.	Injury 1 week after treatment.	Injury 9 weeks after treatment.	Injury 8½ months after treatment.
	Inches.			
1	3	No injury except the killing of water sprout at base of tree.	Crown girdled. No streaking. Foliage strongly purplish in color.	Bark around crown below ground very dark and friable.
2	3	Streak runs up trunk on north side and out onto several limbs. More than half the leaves, especially on north side, badly injured.	Crown girdled. One wide streak and one narrow one run to top of tree. About one-third the leaves killed and others spotted. The rest have a strongly purplish color. Fruit small and highly colored, except on killed limbs, where it is dead and shriveled	Bark at crown not so dark as No. 1 and rather tough instead of friable.
3	3½	Faint streaks run from trunk onto several limbs. About two-thirds the leaves, especially on southeast side, badly injured.[a]	Crown girdled. Streak three-fourths around trunk runs to top of tree. About three-fourths the leaves killed and others spotted. The rest normal in color.	Bark at crown rather dark and more friable than after 9 weeks.
4	3½	Leaves of four spurs on main limb badly injured.	Crown girdled. Wide streak runs to top of tree. About one-fourth the leaves killed or spotted. The rest normal in color.	About like No. 3.
5	6	Faint streaks run from trunk onto limbs. About one-fourth the leaves injured.	Crown girdled. One wide and one narrow streak run to top of tree. About one-eighth the leaves killed or spotted, the rest purplish in color.	Bark at crown very dark and friable.
6	9	Many leaves badly injured..	Girdled at crown and for 18 inches above ground. Several streaks run to top of tree. About one-half the leaves dead and many others spotted, the rest normal in color. Fruit dark and soft at core	Do.

[a] In removing the soil from crown for treatment a slight wound, not through the bark, was accidentally made on west side.

Of much significance in reference to the interpretation of these results (see p. 314) is the change that takes place under ground. On September 26, nine weeks after treatment, the color of the dead bark below and above ground was about the same—namely, about the same shade of brown as a roasted coffee berry. By the following March the color below the ground was a very much darker brown, especially that of No.

1, 5, and 6, while above the ground it had darkened but slightly. A corresponding change had taken place in the texture of the bark. All the dead bark was very tough and fibrous when first killed, and on all aerial portions it remained so, but on the crowns below the surface of the ground it gradually became so disintegrated, especially on those with the darkest color, that it could easily be picked apart with the fingers. When dry, it was rather brittle; when wet, it was so soft as to be almost pasty in certain portions. It seems probable that this disintegration is largely due to the invasion of saprophytic microorganisms rather than to the direct action of the arsenicals.

Comparing the injury through rough bark with that through the smooth bark of the crown, in both cases unwounded, we find that the smooth bark is vulnerable through lenticels and the rough bark through cracks or fissures caused by growth in the cambium region. Either is sufficient to make possible the girdling of the tree or limb at the point of application. Which permits the more rapid absorption depends upon conditions and is, indeed, of little moment.

INJURY THROUGH ROOTS

As most of the spray mixture applied to a tree eventually reaches the ground under it, the likelihood of injury to the roots or to the whole tree through the roots is a subject of interest.

INJURY FROM BANDAGES

A few roots were treated by the bandage method used largely on the branches. The number so treated, however, was relatively small for two reasons: First, the amount of work required is vastly greater than if branches are used, and, second, it is almost impossible to uncover roots and apply bandages to them without wounding them. Most apple roots of a suitable size are covered with fine rootlets the removal of which makes wounds that readily admit the arsenic. However, enough were found free from all side roots to make it possible to establish the following facts: (1) That the unbroken periderm is quite resistant to the absorption of arsenical compounds, (2) that some of the lenticels will admit it, and (3) that when admitted through a wound the effect is about the same as in a branch of the same size. Indeed, we came to regard the susceptibility of roots ½ inch in diameter or larger as about the same as that of branches of equal size.

INJURY BY ABSORPTION THROUGH THE FINE FEEDING ROOTS

In addition to the possibility of a localized injury of crowns or roots by a heavy accumulation of arsenical insecticides that have run down the trunks, we must consider the likelihood of small amounts mingling

with the soil and being taken up in solution with the soil water normally absorbed by the roots. It is natural to suppose that some is absorbed in this way, and Headden's work convinces one that the arsenic thereby accumulated in the tissues of the tree in sufficient amounts may be readily determined by chemical analysis. Whether or not the trees are materially injured by arsenic absorbed in this way is a debatable question.

To determine whether or not a visible injury can easily be brought about in this way, arsenic trioxid was added to the soil under three trees and sodium arsenite was added to the soil under three others. The chemicals were mixed with water in the proportion of 1 to 1,000 by weight. The sodium arsenite was completely dissolved before being applied. The chemicals were sprinkled on the surface of the ground with an ordinary watering pot. Repeated treatments were given, as shown in Table XI. The first application was made to a circular area of ground 8 feet in diameter, with the tree in the center. The second was made in a ring 2 feet wide just outside the first treatment. Subsequent applications alternated between these two areas of ground.

No crop was grown in the orchard, and the ground was harrowed at frequent intervals but not plowed. The trees were irrigated by shallow furrows running about 4 feet from them. The precipitation during the experiment was as follows:

1911.

	Inches		Inches.
August	1.52	November	1.00
September	1.73	December	.65
October	2.15		

1912.

January	0.92	July	1.91
February	.39	August	1.63
March	1.86	September	1.66
April	3.59	October	3.23
May	2.66	November	.37
June	2.69	December	.74

1913.

January	0.78	March	0.44
February	.47	April	1.65

No external symptoms of injury appeared in the aerial portion of any of the trees during the entire course of the experiment. The roots were not examined until the end of the experiment, for obvious reasons. Such injuries as were present in the roots at that time are recorded in Table XI. They were all strictly local, with no suggestion of a general or systemic poisoning.

TABLE XI.—*Results of treating the soil underneath apple trees with arsenical compounds to determine injury from absorption by roots*

No.	Chemical.	Amounts and dates of application.	Injury.
1	Arsenic trioxid ..	1911: Aug. 22 and Sept. 4, 20 gm. 1912: June 18 and 20, July 2, 9, 16, and 30, 20 gm.; Sept. 4, 40 gm.; Sept. 25 and Oct. 18, 80 gm. Total, 360 gm.	Two roots have outer bark slightly injured. A few rootlets killed. Leaves and branches uninjured.
2	.....do...	1911: Aug. 22 and Sept. 4, 20 gm. 1912: June 18 and 25, July 2, 9, 16, 30, and Sept. 4, 20 gm.; Sept. 25, 40 gm. Total, 220 gm.	Leaves and branches uninjured. One root has outer bark injured. A few rootlets killed at the tips.
3	.. ..do..	...do	Leaves and branches uninjured. A few rootlets killed.
4	Sodium arsenite...	1911: Aug. 22 and Sept. 4, 25 gm. 1912: June 18 and 25, July 2, 9, 16, and 30, 25 gm.; Sept. 4, 50 gm.; Sept. 25 and Oct. 18, 100 gm. Total, 450 gm.	Leaves and branches uninjured. Three roots have outer bark covered in places with anastomosing spots that do not extend to the cambium. Many dead rootlets.
5	.....do.....	1911: Aug. 22 and Sept. 4, 25 gm. 1912: June 18 and 25, July 2, 9, 16, 30, and Sept. 4, 25 gm.; Sept. 25, 50 gm. Total, 275 gm.	Leaves and branches uninjured. Bark of roots slightly injured. Many rootlets killed.
6	.... do.....	Same as No. 5, except that on Sept. 25 only 25 gm. were used. Total, 250 gm.	Leaves and branches uninjured. No injury to large roots and very little to rootlets.

In the spring of 1913 it became necessary to remove the entire orchard block containing these trees, otherwise the applications to the soil would have been continued to date or until the trees had been severely injured.

One conclusion is obvious, that considerable quantities of arsenic may be applied to an orchard soil relatively free from alkali without immediate harmful results. It should, perhaps, be added that the orchard in which this work was carried on is relatively free from alkali and that soils rich in certain alkali salts might keep more of the arsenical in solution.

Too much significance must not be placed on the fact that a soluble compound (sodium arsenite) was applied to some of the trees, as we have no assurance that a large proportion was not rendered insoluble by the action of the soil.

SYSTEMIC POISONING

It is a noteworthy fact that the injuries described thus far in this paper were for the most part local. In fact, all were purely local except those that were a result of girdling limbs or trunks.

It is a pertinent question whether or not trees can be made to suffer a general or systemic poisoning from arsenic, and still more pertinent whether or not such a poisoning ever occurs as a result of orchard spraying as now practiced by fruit growers. Headden has described certain symptoms which he suspects are due to systemic poisoning, but he does not regard the evidence as conclusive.

Our own work on this subject has been meager and does not warrant very far reaching conclusions. It has been confined to two lines: (1) The treatment of the soil recorded in Table XI, and (2) observations on trees that have been treated with bandages on nearly every branch so

that almost the entire top of the tree is killed. In the former case, there were no general symptoms of injury during the course of the experiment. In the latter case, with nearly all the smaller branches killed and streaks running down the larger limbs and trunks, the few remaining branches grew vigorously and the foliage appeared perfectly healthy and normal. As a result of so many branches being killed, many buds pushed out below, making succulent water sprouts. It is well recognized that most of the arsenic absorbed through wounds in the bark may be deposited in the wood and bark that are killed and not carried extensively through the tree. That some is carried into the unkilled portions is shown by analyses made by the chemistry department of this Station. The work of Headden has established beyond question that considerable amounts may be taken up from the soil before the tissues are actually killed. All things considered, we think it doubtful if general systemic poisoning has occurred to any considerable extent as a result of orchard spraying, though we have no reason to believe that it can not be produced experimentally.

SIGNIFICANCE OF RESULTS

The results of the foregoing experiments are of interest chiefly in relation to the pioneer work done by Headden, of the Colorado Agricultural Experiment Station, on the crown injury to fruit trees in Colorado and elsewhere and the relation of spraying for codling moth to this injury. Headden's work was reviewed in the opening pages of this paper, and certain points of strength and of weakness in his evidence were pointed out.

In this contribution we have shown that arsenical compounds used for spraying can penetrate the corky layer of the rough bark and cause a corrosion and killing of the inner bark and produce symptoms similar to those described by Headden. The symptoms we have produced should be closely compared with those found in Colorado. They are alike in several respects: (1) The bark is killed and discolored in a localized area at and extending out from the point in contact with the arsenic; (2) the wood underneath the dead bark is discolored in an irregular region growing smaller toward the center of the tree (if there is no blackheart from winter injury) and up and down from the point of injury; (3) the dead bark, if in contact with moist soil, becomes darker, more or less disintegrated, and friable; (4) the fruit on trees with corroded crowns may become small and highly colored; (5) in early fall the foliage may take on a distinctly purplish color. There are two points of difference: (1) There is a marked tendency for longitudinal dead streaks to be formed as a result of artificial treatment; (2) we have never produced corroded crowns that were quite so dark in color or quite so friable in texture as those on some of the injured trees in Colorado.

These comparisons are worthy of further analysis. There is, on the whole, a rather striking similarity if we restrict our comparisons to injuries on the thick, rough-barked crowns, trunks, and limbs, where the treatment was not too severe and the injury was correspondingly slow. The streaking effects were produced as a result of very rapid absorption, either through wounds or thin places in the bark, and a very close similarity need not be expected. It would appear that up to a certain limit the slower the injury and the longer the killed bark remains in contact with the moist earth the darker and more friable it becomes. It is possible, therefore, that if the injury in the Colorado orchards under consideration has been slowly developing on old trees for a considerable number of years this would account for the difference. As for the small and highly colored fruit, the yellowish foliage, and the restricted terminal growth, it is well known to horticulturists that these conditions may follow a nearly complete girdling of the crowns from any cause whatsoever. The purplish color of the foliage noted in some cases (Table X) late in the season both on our experimental trees and in some of the Colorado orchards is not restricted to arsenical injury. At the time it appeared in our treated trees it could be found in untreated ones where small branches had been partly broken down or the bark scraped off by farm machinery.

Realizing the difficulty of making comparisons of symptoms from written descriptions and the importance of such studies where the etiology of a disease is unknown, we made, in 1910, a rather extended trip into the affected districts of Colorado and Utah. On this trip we received most courteous consideration and many helpful suggestions from members of the Experiment Station staffs in these two States, especially from Headden and Ball. We found that the extent of injury suffered by the orchards was not overstated in Headden's bulletins and that the symptoms were most accurately described.

One additional feature impressed us very much—namely, the amount of injury done the aerial portions of the trees relative to the size of the corroded area on the crown. To be sure, many trees were entirely girdled and others nearly so, but some trees 8 to 12 inches in diameter had a dead patch of bark on the crown no larger than a man's hand and yet showed in fruit, foliage, and terminal growth a degree of injury wholly unexpected if one judges by the trees, frequently encountered, that had the crowns more or less injured by mice, blight, or other agencies. We dug up a few of the sick trees completely and uncovered the crowns and upper roots of a great many others. We did not dig up all, as this would have been a herculean task which time did not permit. The only possibility that some of the trees recorded as having only a small dead patch on the crown could have had other injuries is that such injuries were on the deeper roots below the attachment to the crown. However,

in no case of trees entirely removed was this region affected except in those having the upper portion of the crown extensively killed and the dead area extending down underneath. This disproportionate injury of crown and branches suggested a systemic poisoning in addition to the effect brought about by girdling.

A considerable search was made for evidence of parasites. Repeated examinations were made with a good traveling microscope, and material for cultures was taken back to the laboratory at Bozeman. A very small proportion of the crowns was found to be attacked by the blight organism *Bacillus amylovorus*, but these were easily distinguished from the others by field observation. A few cases differed from the blight and from the crown injury more commonly found in that the dead bark was tougher, juicier, and a more red-brown in color with a distinctly stratified appearance. By microscopic examination no microorganisms were found in these cases, and cultures from the inner bark yielded nothing. Our efforts to find parasitic fungi or bacteria in the more common form of the trouble, either by microscopical examination or by culture, yielded no positive results, though some saprophytes were found, as might be expected. We would, however, warn the reader against concluding from this evidence that the possibility of a parasitic agent was eliminated, for all experienced plant pathologists are aware that much more extended work would be required to justify such a contention.

Our observations on this trip brought no evidence against the arsenical theory. On the other hand, the contention of Ball and others (1, 2) that this trouble is caused by alkali and seepage water appeared wholly untenable, as many of the trees were in positions where such conditions were absent and there seemed to be no especial prevalence of crown injury where they occurred.

It is apparent from Ball's publications that he was confusing two troubles sharply separated by Headden, who alleged them to be due, respectively, to arsenical poisoning and to an excess of nitrates in the soil (6, 8, 9). That the latter may be due to alkali and seepage is debatable, but that is not within the scope of this paper.

We have established conclusively that arsenical compounds used as insecticides can be made to injure the crowns of trees under conditions very similar to those that result from some orchard practices. Whether or not such compounds are responsible for killing the trees under discussion in Colorado or elsewhere, or any portion of them, is quite a different question, and each reader will form his own opinions from the evidence.

We wish here to emphasize the importance of making further studies to eliminate or establish other factors as causative agents for this trouble, especially parasitic organisms and climatic conditions. The former possibility has been hardly touched so far as Colorado is concerned, though many brief studies of forms of "collar-rot" and "crownrot"

have been made elsewhere, with varying results. The latter possibility has received some consideration from Grossenbacher (3, 4) especially, but we are still left in doubt as to the application of his work to conditions in the Rocky Mountain region.

On the part of some investigators of crown injuries of fruit trees, there seems to be a tendency to take a very narrow view of the field and to ascribe to some one cause a number of troubles characterized by injury to the bark on or near the crown. That such conditions are caused by different agencies is well established, and it seems highly improbable that we will make much headway by trying to discredit the work of others in support of a narrow hobby.

CONCLUSIONS

(1) The periderm on the smooth bark of apple trees is practically impervious to arsenical solutions.

(2) If such solutions are admitted during the growing season, more or less injury will follow.

(3) The solutions may be admitted through wounds, lenticels, or latent buds.

(4) As the trees become older and rough bark is produced, the cracks made in its production will admit arsenical solutions to the inner tissues, which will be injured.

(5) Roots and branches of equal size and with similar bark are about equally susceptible to arsenical injury.

(6) If the injury is rapid, for example, the arsenic gaining entrance through a wound in the smooth bark, very definite longitudinal streaks will be produced in the bark and sapwood.

(7) If the injury is slow, the arsenic gradually entering through cracks of the normal rough bark, such streaks are very little in evidence.

(8) Paint applied to fresh wounds offers a partial, but not complete protection.

(9) Of the arsenical compounds used as insecticides, calcium arsenite is the most injurious when admitted to the inner bark.

(10) The injury is not necessarily due to impurities in the arsenical compounds.

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(6) ———
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(7) ———
1910. The fixation of nitrogen in some Colorado soils. Colo. Agr. Exp. Sta. Bul. 155, 48 p., illus.
(8) ———
1910. Nitrates in the soil. Colo. Agr. Exp. Sta. Bul. 160, 8 p.
(9) ———
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(10) SWINGLE, D. B., and MORRIS, H. E.
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(11) WHIPPLE, O. B.
1907. Western Slope fruit investigation, 1906. Colo. Agr. Exp. Sta. Bul. 118, 16 p.

PLATE 79

Funnel used in the treatment of trunks with arsenical solutions and suspensions. × ⅙.

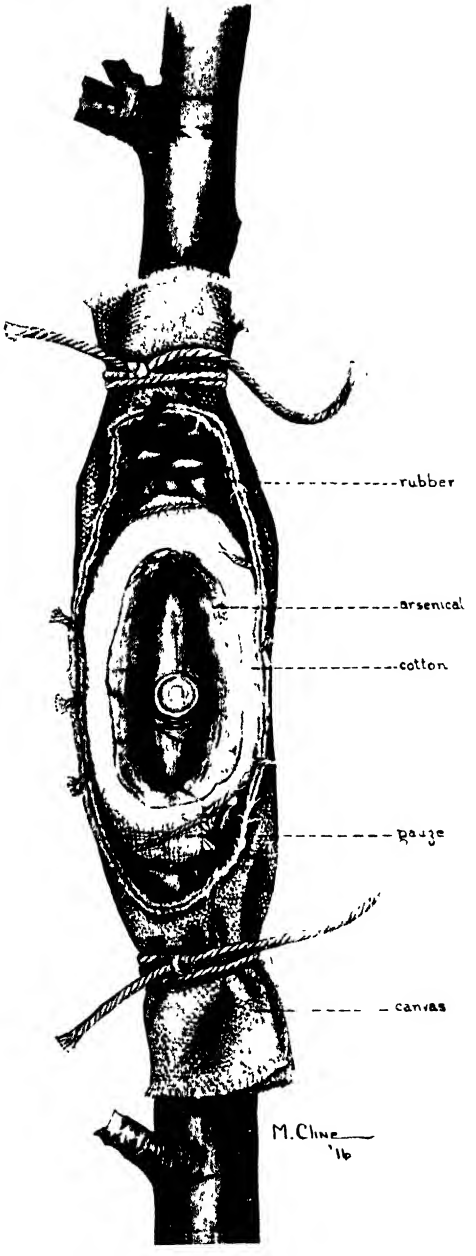

rubber
arsenical
cotton
gauze
canvas
M. Cline
'16

PLATE 80

Dissection of bandage used in the treatment of branches with insoluble arsenical compounds. Those used in treatment with soluble compounds were the same, except that the chemical was in solution and absorbed by the cotton. × ⅔.

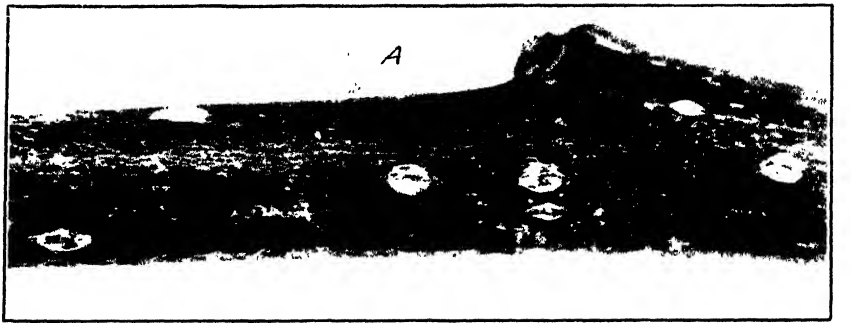
PLATE 81

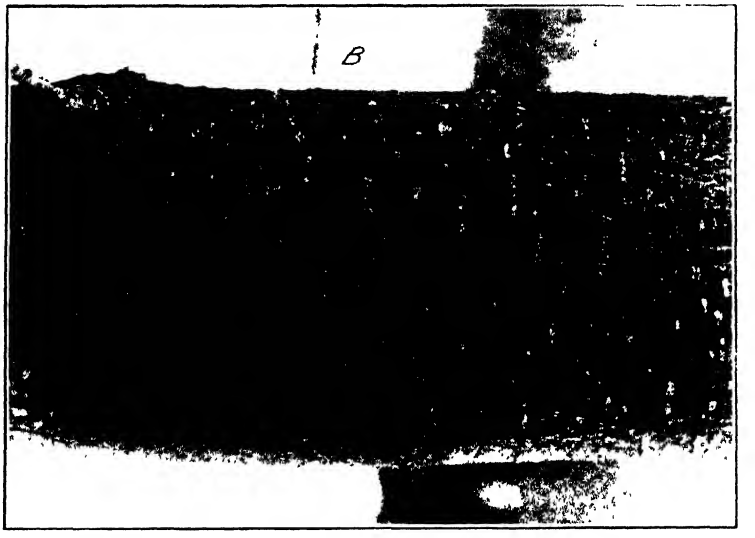
Character of rough bark of apple tree.

A.—First splits in the formation of rough bark. × 2/3.
B.—Roughened condition as found on practically all old trunks and limbs. × 2/5.

A

B


A


B

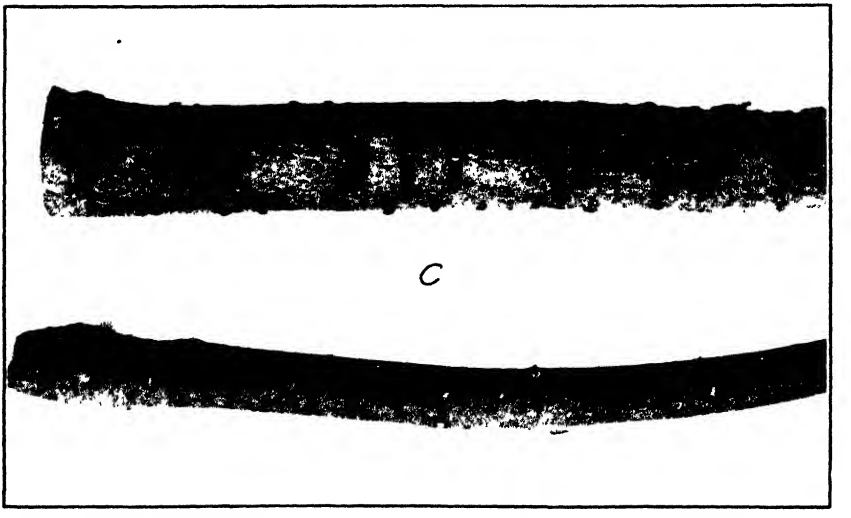

C

PLATE 82

Lenticels of different ages.

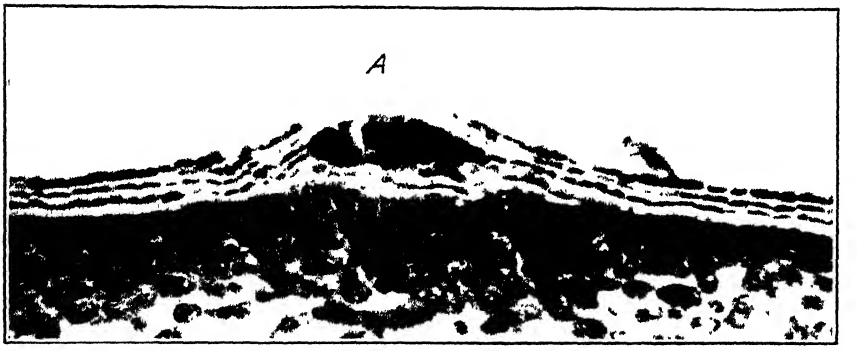
A.—Lenticels on a young shoot that has just completed the season's growth. × 6.
B.—Lenticels on a limb about 8 years old. × ⅞.
C.—Lenticels on roots. × 1.

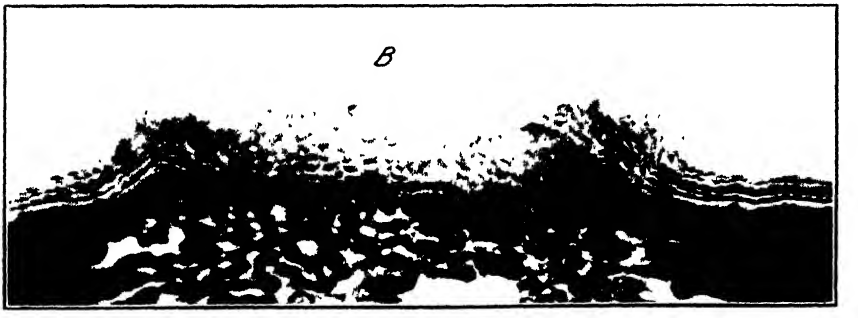
PLATE 83

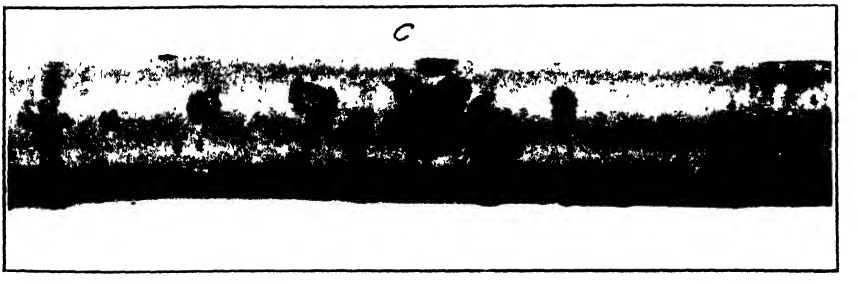
A.—Section through a lenticel just forming. × 80.

B.—Section through a well-developed lenticel. × 80.

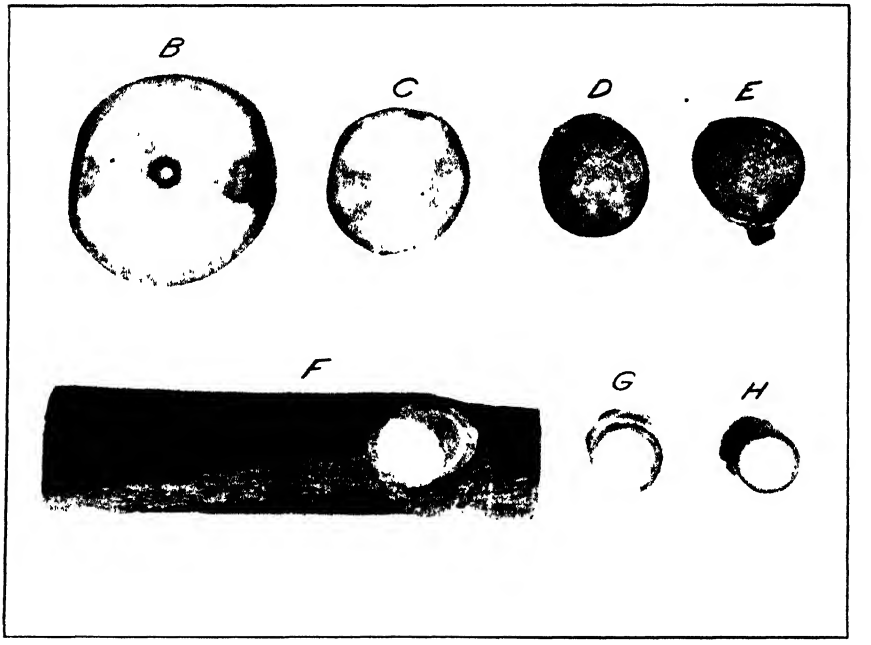
C.—Lenticel spots formed by the absorption of zinc arsenite through lenticels inclosed in arsenical bandages. × 1.


A


B


C

A


B
C
D
E
F
G
H

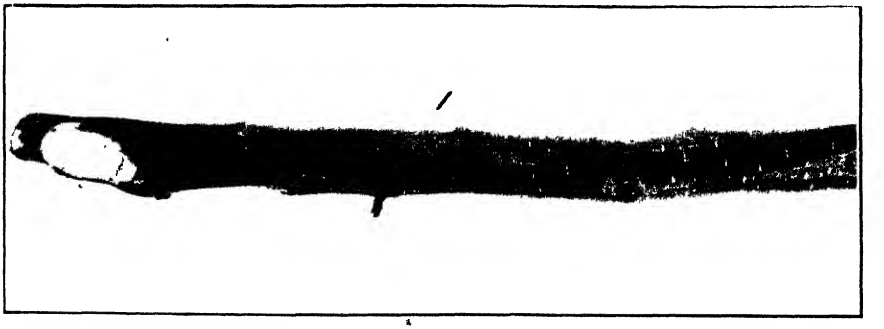

I

PLATE 84

Character of arsenical injury.

A.—Branch treated with a solution of arsenic acid. The portion photographed was inclosed by the bandage. Bark removed for photograph. The knot in the dark, injured streak was made by cutting off a limb just before the solution was applied. The other knots shown in the figure were old and showed no arsenical injury. × 1.

B to I.—Portions of tree injured by a single treatment with sodium-arsenite solution. There were two fresh wounds under the bandage on opposite sides of the limb.

B.—Cross section of main limb 1 foot below intersection with treated branch.

C.—Cross section 16 inches below bandage and just above main limb. × 1.

D.—Cross section under bandage just below wounds. × 1.

E.—Cross section 1 foot above bandage. × 1.

F.—Surface view 8 to 11 inches below bandage. × 1.

G and H.—Cross sections of side branch that joined treated branch 8 inches below bandage. The wound made by its removal is shown in figure F. Figure G is 1 inch and figure H is 6 inches above the intersection with the treated branch. × 1.

I.—Main limb with treated branch removed. The wound made by its removal is shown near the left end of the figure. × ¼.

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A SQUASH DISEASE CAUSED BY CHOANEPHORA CUCURBITARUM[1]

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INTRODUCTION

It was noted during the past season (1916) that summer squashes in the vicinity of West Raleigh, North Carolina, were attacked by a phycomycetous fungus, *Choanephora cucurbitarum* (B. and Rav.) Thaxter. Attention was directed to this fungus primarily because of the luxuriance of its fructifications on affected parts of squashes (*Cucurbita* spp.) and of the rapidity with which it destroyed such parts. A study was therefore made of the development of the disease and of the morphology of the causal organism. Conidial, sporangial, chlamydosporic, and zygosporic stages have been developed, and there have been made certain observations which are at variance with previously recorded facts relative to *C. cucurbitarum* and other species of the same genus. It is the present purpose, therefore, to record the results of this study as additional knowledge of this exceptionally interesting form and to indicate its importance as a parasite on the squash plant.

HISTORY AND DISTRIBUTION OF THE FUNGUS

This fungus has long been known to occur within the United States, having first been described in 1875 by Berkeley[2] as *Rhopalomyces cucurbitarum* from decaying squashes collected in South Carolina by Ravenel. The organism has subsequently been found in other States, as noted by Thaxter[3] in his account of the genus Choanephora. Dr. Thaxter himself collected the fungus on squashes at Waverley, Massachusetts, and on cultivated species of Hibiscus and a wild malvaceous plant at Eustis, Florida. He records the fact that the same plant is in the Curtis Herbarium at Harvard University under the name "*Aspergillus cucurbiteus*, Hillsborough, N. C., on squashes." He further examined specimens of the same fungus on squashes sent from New York by Prof. Peck and from Ohio by Prof. Morgan.

[1] Published with the permission of the Director of the North Carolina Agricultural Experiment Station.

[2] Berkeley, M. J. Notices of North American fungi. *In* Grevillea, v. 3, no. 27, p. 109. 1875.

[3] Thaxter, Roland. A New England Choanephora. *In* Rhodora, v. 5, no. 52, p. 97–102, pl. 46. 1903.

Clinton,[1] in 1903, reported the occurrence of the disease in Connecticut, and included photographic illustrations of the fungus on squash flowers. This species has also been noted in Brazil on the petals of a species of Hibiscus and on certain other plants, whence it was described by Möller[2] as *Choanephora americana*.

Numerous collections have been made during the past summer in the vicinity of West Raleigh, N. C., and in two other localities within the State,—Winston-Salem and Walnut Cove. Since these points are rather widely separated, it is believed that *Choanephora cucurbitarum* is generally present throughout the State. From reports received, unaccompanied, however, by specimens, there seems to be no doubt that the disease was present during the past season in other Southern States.

HOSTS OF THE FUNGUS

During the past summer the disease was found on several varieties of *Cucurbita pepo*, being most destructive on the "pattypan" types of summer squashes, commonly known as cymlings. The fungus has also been found on the fading flowers of cucumber (*Cucumis sativus*) althea (*Hibiscus syriacus*), scarlet hibiscus (*Hibiscus coccineus*), okra (*Hibiscus esculentus*), and cotton (*Gossypium herbaceum*). It appears to be parasitic, however, only upon the squash. Previous investigations, as has been indicated, reported the occurrence of *C. cucurbitarum* on squashes, *Hibiscus* spp., a wild mallow, and certain other plants.

ECONOMIC IMPORTANCE

The disease is probably not the cause of appreciable damage to squashes annually, but appears in epidemic form only under certain meteorological conditions. Conditions of high humidity and excessive rainfall were generally prevalent in the Southern States during the past summer, and are believed to be correlated with epidemics of the squash disease. At no time during the four preceding summers has the disease been sufficiently destructive to attract the writer's attention.

Since squashes are not a crop of any considerable financial importance to the market gardener in North Carolina, it is especially difficult to obtain figures on the loss due to this disease. The crop is very generally grown in home gardens, and even though a considerable proportion of the fruits decay, an ample crop for home use remains. All of the flowers have been observed to be blighted and all of the fruits destroyed on certain vines. Thaxter,[3] in discussing the injury by *C. cucurbitarum*, says: "It is undoubtedly often responsible for the destruction of no inconsiderable percentage of the crop." Clinton[4] states that "it no doubt often blasts the blossoms and may rot the fruit, also."

[1] Clinton, G. P. Report of the botanist. Diseases of plants cultivated in Connecticut. *In* Conn. Agr. Exp. Sta. 27th Ann. Rept., [1902]/3, p. 359, pl. 26. 1904.

[2] Möller, Alfred. Phycomyceten und Ascomyceten. p. 18, pl. 1, fig. 1-4. Jena, 1901. (Schimper, A. F. W. Botanische Mittheilungen aus den Tropen. Heft 9.)

[3] Thaxter, Roland. Op. cit.

[4] Clinton, G. P. Loc. cit.

APPEARANCE OF THE DISEASE

The symptoms of the disease on flowers and fruits of squashes is quite characteristic. The fungus covers the affected parts with a luxuriant crop of conidiophores, with no evidence of any vegetative mycelium. The fructifications of two other fungi, *Rhizopus nigricans* Ehr. and *Botrytis vulgaris* Fr., may appear on the surface of decaying squash fruits, but neither of them possesses the metallic luster which characterizes the fructifying filaments of *C. cucurbitarum.*

The first evidence of attack on the flowers usually appears on the day following the opening of these flowers. Early in the morning of the second day, the fungus will have reached its most luxuriant development, especially immediately after a shower, and the fading flowers will be covered by a dense coating of immature, white conidial heads. Within a few hours these fructifications will have matured and will have changed in color from white to brown and at length to purplish black. When staminate flowers are attacked, the mycelium may pass downward into the flower pedicel, converting the tissues into a soft, translucent condition. Abscission of the flowers generally occurs before the pedicels become invaded. The appearance of the disease on flowers and flower pedicels is shown in Plate 87, *A*.

When pistillate flowers are attacked, the fungus passes from the fading flower into the young squash. Incipient decay from infection through the corolla is shown in Plate 87, *D*, as is also complete decay involving the fruit in a soft, wet rot with a profuse envelopment of conidial branches. The decay of fruits progresses with remarkable rapidity, as is shown in Plate 87, *B*, *C*. The photographic exposure for figure *B* was made about 15 hours after the one for figure *C*, the squash, meanwhile, having been kept in a moist chamber. Occasional fruits were found in which the fungus appeared not to have gained entrance through the corolla.

ETIOLOGY AND DEVELOPMENT OF THE FUNGUS

Choanephora cucurbitarum, the cause of this squash disease, is unique in that it is the only known American representative of the family Choanephoraceae. Two other species have been described from India, one *C. infundibulifera* (Curr.) Cunn. on the flowers of *Hibiscus rosa-sinensis* and the other, *C. Simsonii* Cunn. on *Ipomoea rubro-coerulea* and *Zinnia elegans*. The former of these was described by Currey [1] in 1873 as Cunninghamia, a new genus of mucedinous fungi, and was referred by Cunningham [2] in 1879 to the Mucorineae.

[1] Currey, Frederick. On a genus in the order of Mucedines. *In* Jour. Linn. Soc. [London], Bot., v. 13, p. 333–334, pl. 7. 1873.

[2] Cunningham, D. D. On the occurrence of conidial fructification in the Mucorini, illustrated by Choanephora. *In* Trans. Linn. Soc. London, s. 2, Bot., v. 1, p. 409–422, pl. 47. 1879.

CONIDIAL STAGE

Observations on the development of the conidial stage agree essentially with those of Thaxter.[1] The erect conidiophores, whose comparative size is shown in Plate 87, *A*, are whitish at first, but at maturity change to a distinct metallic luster. They are broadest at the upper extremity, which becomes dilated into a capitate vesicle. From this capitulum, a few to a dozen or more ramuli arise, which in turn become vesicular (Pl. 85, *A*). The ramuli commonly remain simple, but occasionally branched ones are present. By a process of budding, an early stage of which is represented in Plate 85, *B*, the capitella become covered with a densely compacted layer of conidia (Pl. 85, *D*). The conidia vary in outline from oval to elliptical and are conspicuously marked by longitudinal striations (Pl. 85, *C*), some of which anastomose. They vary in size from 15 to 25 by 7.5 to 11 μ and in color from light brown to reddish brown. The base of each conidium is provided with a hyalin appendage, the adherent portion of the sterigma. When the ramuli are broken off, scars as shown in Plate 85, *E*, are evident on the primary vesicle.

Variations from this normal course of development have been noted. Conidia may arise directly from the surface of the primary head, a condition noted both by Thaxter [1] and myself and which is characteristic of the genus Rhopalomyces. Occasionally the conidiophore branches and gives rise to two primary heads on each of which a layer of conidia is formed. These are not necessarily depauperate forms, since they appear in cultures with normal well-developed conidial fructifications.

GERMINATION OF CONIDIA

Germination readily follows when the conidia are sown either on nutrient agar, such as bean, potato, glycerin, or cellulose agar, or in drops of water. Cunningham [2] states that germination of conidia of *C. infundibulifera* does not occur in water. Germination of *C. cucurbitarum* does not proceed, however, with equal rapidity in water and in nutrient agar, as shown by Plate 85, *G*, *H*, the former of which represents growth on nutrient agar 3½ hours after the commencement of cultivation, and the latter growth in water, after an equal period. Conidia were obtained from the same source in both cases and were germinated under the same conditions of light and temperature.

The first evidence of germination is the considerable increase in size which takes place before the emergence of the germ tube. Typically a single lateral germ tube is formed, occasionally two, which in some cases arise from the extremity. As a rule, the germ tubes will have emerged within two hours after sowing. The rate of subsequent development is indicated in the stages represented in Plate 85, *I*. Plate 85, *I*, *a*, shows

[1] Thaxter, Roland. Op. cit. [2] Cunningham, D. D. Op. cit.

an early stage in the development of a hypha from each of two conidia which were kept under constant observation and outlined at 15-minute intervals, with the aid of a camera lucida. Each successive stage is thus 15 minutes older than the preceding one, making an interval of an hour between Plate 85, *I*, *a*, and Plate 85, *I*, *e*. Under favorable conditions of temperature (room temperature during July and August) the mycelium will have profusely covered a 10-cm. nutrient-agar plate within 24 hours and will have fruited luxuriantly. Thaxter[1] found that this fungus fruits abundantly in a moist chamber when cultivated on squash tissues, but that fructifications very rarely appear on potato agar and similar nutrients in tubes.

SPORANGIAL STAGE.

The sporangia of *C. americana*, which is regarded as synonymous with *C. cucurbitarum*, was described, as has been indicated, by Möller.[2] Thaxter states that he was never able to obtain sporangia of this organism, although he repeatedly cultivated it under unfavorable conditions of nutrition, which were held by Cunningham[3] to favor their production in *C. infundibulifera*. Such unfavorable conditions are present when the artificial medium is exhausted by the vegetative growth of the fungus and when the mycelia developed from conidia produced in artificial culture. When grown under artificial conditions, *C. infundibulifera* appeared to lose its vigor progressively, since no reproductive structures developed in cultures the third generation removed from the natural host. Neither were sporangia formed on the host in the natural state, a condition similar to that found by the writer with *C. cucurbitarum*. In fact, the conidial stage only has been found on squashes, the other stages having all appeared in culture.

Sporangia generally appear with conidial fruits, but have been formed alone on one set of cellulose-, bean-, and glycerin-agar plates which were continuously kept in the dark. Other series of cultures on the same kind of media when grown in darkness developed a few of both types of asexual spores. A different strain of fungus was used in these series. Certain cultures on cellulose agar, when kept in the light, developed only sporangia. Evidently light alone is not the determining factor in the formation of conidia and sporangia in *C. cucurbitarum*. Cunningham[3] never obtained sporangia apart from the conidial form.

Mature sporangia are present as early as mature conidia. They usually form most abundantly at the center of the colony on plate cultures. Sporangia are first evident as pendent, white, globular enlargements The sporangium becomes separated from the sporangiophore by a globular columella, which can best be seen when mature sporangia are burst (Pl. 85, *L*). At maturity the sporangia are black and vary in diameter from 35 to 160 μ. Diminutive sporangia have as few as two or three

[1] Thaxter, Roland. Op. cit. [2] Möller, Alfred. Loc. cit. [3] Cunningham, D. D. Op. cit.

spores. The spores are ovoidal or elongated rarely inequilateral and of the same color as the conidia. They are somewhat larger than the conidia, being 18 to 30 by 10 to 15 μ. They lack the striations which characterize the conidia, but are unique in that they are provided with two or three terminal or lateral hyalin appendages, from each of which a tuft of 12 to 20 hairlike processes extend (Pl. 85, *N*). These ciliate processes are from 1 to 1½ times as long as the spores.

GERMINATION OF SPORANGIOSPORES

The germination of these spores is quite similar to that of conidia Within two or three hours after the spores are placed in nutrient media there is an increase in size of the spores, followed by the emergence of one or two lateral germ tubes (Pl. 85, *J*). Within a few hours a profusely branched mycelium is formed, which in 24 to 48 hours has matured a crop of sporangia and conidia.

CHLAMYDOSPORES

The mycelim in 4- to 5-day-old cultures on nutrient agar is commonly septate. Not infrequently well differentiated chlamydospores, as shown in Plate 85, *O*, will have formed within this time. In cultures 2 weeks old chains of chlamydospores, as represented in Plate 85, *M*, are sometimes developed. No chlamydospore formation occurred in the form which Thaxter [1] had under observation. These bodies have never been observed to germinate.

ZYGOSPORIC STAGE

Cunningham observed zygospore formation in the two Indian species of Choanephora, and his account of the development of *C. infundibulifera* [2] contains a detailed description of this process. Zygospores of *C. cucurbitarum* appear not to have been observed prior to the present study. No noteworthy differences are presented by the American species when contrasted with the Indian forms. A club-shaped branch first arises from each of two adjacent mycelial filaments. These gradually enlarge and become densely filled with granular protoplasm. The contents tend to accumulate in the apical extremity, and a septum forms, separating the gamete from the suspensor. When two of these branches are situated sufficiently close together, their bowed tips come in contact and the gametes become flattened along the opposed surfaces. The partition is then absorbed and the two gametes fuse. Such a stage is represented in Plate 86, *B*, *C*, at which time the two saccular suspensors are quite clear and empty as contrasted with the immature zygospore, which is convex on the upper side and is filled with coarse granules and numerous yellowish oil globules. As the zygospore matures, this convexity

[1] Thaxter, Roland. Op. cit. [2] Cunningham, D. D. Op. cit.

is increased until the terminal walls of the suspensors tend to be in the same plane (Pl. 86, *E*), and the suspensors appear to support it from one side. Meanwhile the exospore has thickened, become dark brown in color, and the oil globules have fused to make one large globule, which occupies one-third to one-fourth of the volume of the ripe zygospore. Zygospores vary in size from 50 to 90 μ in the longest diameter.

Mature zygospores were first observed in nutrient agar cultures 48 hours old which had been planted with conidia taken from squash flowers. They appeared, however, in only a small number of isolation cultures prepared in this way. They are formed embedded within the agar and on its surface and can be seen with the unaided eye as numerous black specks when the isolation plates are held against a white background. Conidia and sporangia are always present in these plates. All attempts to germinate these zygospores have thus far met with failure. Furthermore, all attempts to determine the conditions necessary for zygospore formation have been futile. Curiously, no zygospores appeared in subcultures from cultures in which zygospores were present; nor have they ever been noted in culture except when the conidia for making these cultures were taken directly from the host plant. It was presumed that this might be due to a loss of vigor in artificial culture. It will be recalled that cultures of *C. infundibulifera* three generations removed from the natural host failed to form asexual spores. No such failure to form conidia and sporangia appears to occur in *C. cucurbitarum*, since it has been grown for 15 generations with no resultant decrease in the luxuriance of its asexual fructifications. Numerous cultures have been obtained from single conidia and sporangiospores with the view of determining whether in this species zygospore formation was conditioned by heterothallicism. Thus far, however, no evidence of this separation of sexes has been found.

When material for studying zygospore development was at hand, the press of other duties prevented the making of a detailed study of the process. Subsequently, it has been impossible to develop zygospores. However, several blocks of agar containing zygospores were fixed, sectioned, and stained. Unfortunately, only a few of the stages were exhibited in these sections. Plate 86, *A*, represents an early stage in which the gametes have been separated from the suspensors. Gametes, suspensors, and vegetative mycelium (Pl. 86, *D*) are found to be multinucleate. It is hoped subsequently to be able to furnish an account of the conditions necessary for zygospore formation and of the cytological phenomena accompanying this process.

Since no complete account of all of the spore forms of this organism has ever been given, it seems desirable to present herewith a brief technical description, together with its synonomy and the known list of hosts.

Choanephora cucurbitarum (Berk. and Rav.) Thaxter

Choanephora cucurbitarum (Berk. and Rav.) Thax., 1903, *in* Rhodora, v. 5, p. 97–108, pl. 46.
Rhopalomyces cucurbitarum Berk and. Rav., 1875, *in* Grevillea, v. 3, no. 27, p. 109.
Rhopalomyces elegans, var. *Cucurbitarum* Marchal, 1893, *in* Rev. Mycol., ann. 15, no. 57, p. 11.
Aspergillus cucurbiteus Curtis, in Harvard Herbarium.
Choanephora americana Möller, 1901, Phycom. u. Ascom., p. 18, pl. 1, fig. 1–4.

Sporangia pendula, globosa, solitaria, polyspora, nigra, 35–160 μ diam.; stipite nitido-violaceo; columella globosa; sporis ovoideis vel ellipsoideis, levibus, brunneo-rufis 18–30×10–15 μ. utrimque interdum atque lateribus penicello ciliorum auctis; ciliis 25–45 μ longis. Condidiophoris usque 6 mm. longis, nitido-violaceis; apice vescicula unica vel pluribus capitatis muriculatis terminatis; conidiis in vescicula capitatim insertis ovoideis longitrorsum striatis, brunneo-rufis, appendicula basilare hyalina, 15–25×7.5–11 μ. Chlamydosporae globosae vel oblongo-ellipsoideae. Rami zygosporae arcuati, zygosporis inaequilateralibus, unico globulo olei genente, 50–90 μ diam.

Hab. in fructibusque floribus vivis Cucurbitae peponis ac in floribus languidis Hibisci esculenti, H. coccinei, H. syriaci, Gossypii herbacei, Cucumis sativii aliisque malvacearum, Americae Bor., atque in petalis adhuc stantibus Hibisci aliisque plantae partibus dejectis, Blumenau brasiliae.

PATHOLOGICAL ANATOMY

Diseased tissues from squash fruits and flower pedicels were fixed, sectioned, and stained for this portion of the study. Mycelium was found to be present in all of the tissues that were involved in softrot or wetrot, with no evidences of disintegration in advance of the mycelium. It is both intercellular and intracellular, as shown by Plate 85, *F*, *K*, figure *F* representing the invaded tissue of the flower pedicel and *K* that of squash fruit. Parasitism appears to be of the necrotic type, with little or no transformation of the cell wall substance. Growth of the fungus on cellulose agar gave no evidence of the secretion of cellulase. Evidence of the presence of cellulase and cytase was further sought, with negative results in 14-day-old cultures of *C. cucurbitarum* on sterilized squash fruits. The fluid from these cultures was filtered through a Chamberland filter and pieces of raw fruits were introduced under aseptic conditions into this sterile filtrate.

DISSEMINATION OF THE FUNGUS

A microscopic examination of the inner surface of the corolla tube and of the filaments of the stamens revealed the fact that conidia of *C. cucurbitarum* are commonly present in open squash flowers. Thirty of the 35 flowers which had opened on the morning of July 6, and which were examined in the afternoon of that day, contained conidia. On the afternoon of the 7th, conidia were present in all the flowers examined,—27. On the 8th, 7 flowers were picked before 7 a. m., and all had conidia within them. On the 10th, conidia were present in 25 flowers which had not yet closed. Since various species of bees so commonly visit squash flowers, a limited number were examined to determine whether they were instrumental in distributing the conidia. The bees, when captured among the

squash plants, were placed in test tubes washed in a small quantity of water, and the wash water was examined by placing drops of this water on a microscopic slide. Conidia were quite commonly present in this water. The striped and spotted cucumber beetles (*Diabrotica vittata* and *D. punctata*, respectively) were also found to be carriers. The wind is also an agent in the dissemination of this fungus, as determined by a series of 13 agar plates which were exposed in a field of squashes on the morning of July 11. On the following day *C. cucurbitarium* had developed on all of these plates.

SUMMARY

(1) *Choanephora cucurbitarum* is parasitic upon summer squashes, causing a blight of the flowers and a rot of the fruits.

(2) The disease has been the cause of very considerable losses during the past summer.

(3) Infection of fruits occurs, for the most part, by the passage of the fungus from the fading corolla to the young squash.

(4) Various species of bees, striped and spotted cucumber beetles, and currents of air are agents of dissemination of the conidia.

(5) The fungus has been found on languid flowers of cucumber, althea, okra, cotton, and other malvaceous plants.

(6) *C. cucurbitarum* is the only known American species of this genus.

(7) The conidial stage alone appears on affected parts of the several host plants.

(8) Sporangial, chlamydosporic and zygosporic stages have been developed in artificial culture. None of these stages have previously been reported by investigators within the United States.

(9) All of the reproductive stages have been noted in the same culture on nutrient agar. Conidia, sporangia, and zygospores may mature in cultures 24 to 48 hours old. No decrease in luxuriance of asexual fructification has been found in culture on nutrient agar.

PLATE 85

Choanephora cucurbitarum:

A.—Immature conidiophore with ramuli developing on the primary vesicle. ×100.

B.—Budlike processes the beginning of conidial formation on the capitellum. ×100.

C.—Conidia. ×250.

D.—Mature capitulum covered with a layer of conidia. ×125.

E.—Primary vesicle from which ramuli have been detached. ×100.

F.—Mycelium in tissue of squash flower pedicel. ×75.

G.—Germination of conidia 3½ hours old in nutrient agar. ×250.

H.—Germination of conidia 3½ hours old in water. ×250.

I.—*a*, Early stage in germination of conidia in hanging drop; *b*, growth of mycelium from same conidia as figure *a* drawn 15 minutes later; *c*, one-half hour later than figure *a*; *d*, 15 minutes later than figure *c*; *e*, one hour later than figure *a*. ×250.

J.—Germination of sporangiospores. ×250.

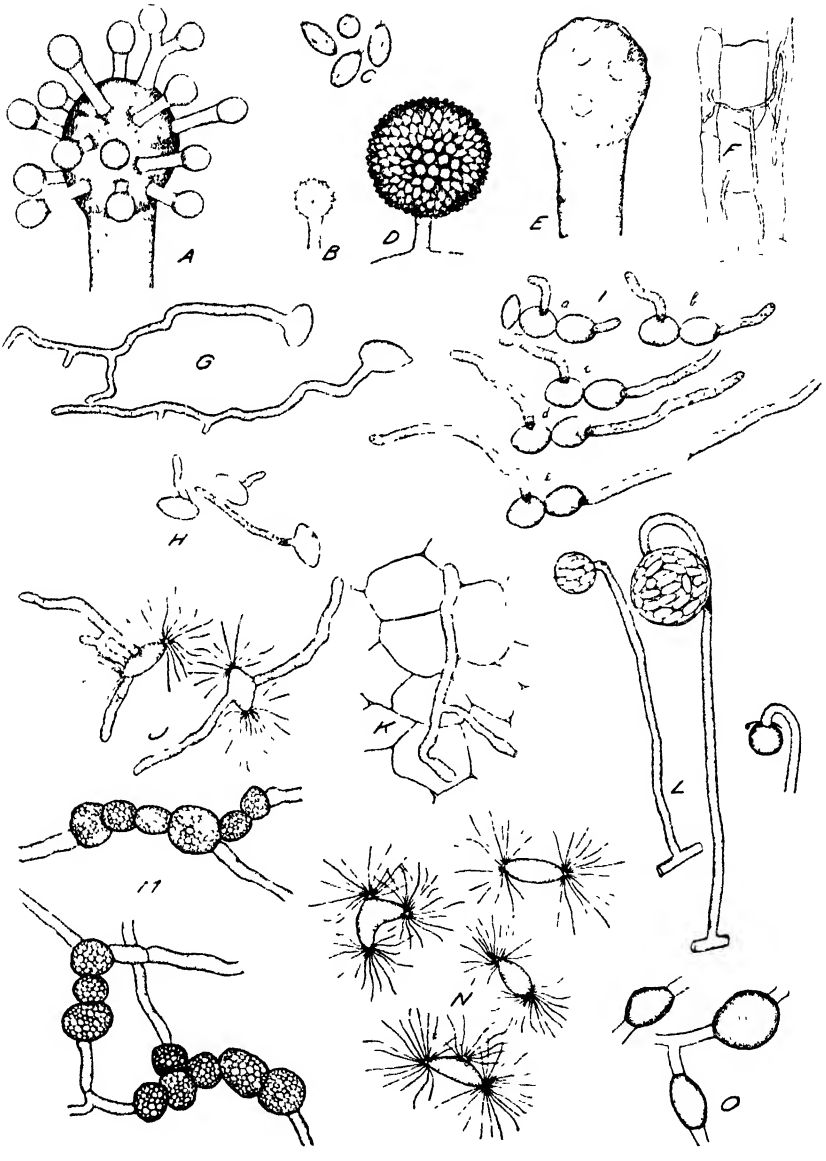
K.—Mycelium of the fungus within the tissues of squash fruit. ×250.

L.—Variation in size of sporangia and columella. ×70.

M.—Chlamydospores borne in chains in old cultures. ×250.

N.—Sporangiospores with tufts of hairlike appendages. ×250.

O.—Chlamydospores formed singly in 4-day-old cultures. ×250.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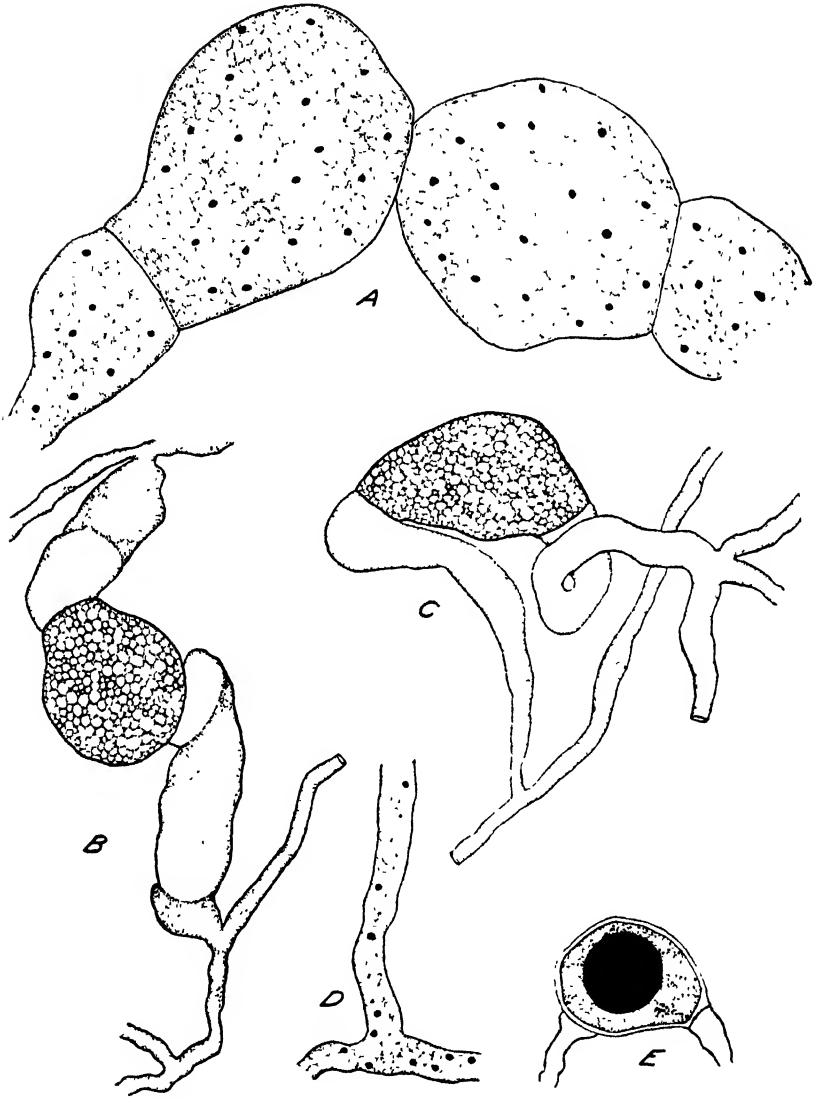
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PLATE 86

Choanephora cucurbitarum:

A.—Gametes prior to fusion. Gametes and suspensors are plurinucleate. ×900.

B.—Young zygospores whose exospore has not yet developed and whose contents are a dense mixture of granular protoplasm and small oil droplets. The origin from two mycelial branches is also shown. ×320.

C.—Lateral view of a young zygospore in the same stage of development as figure B. ×320.

D.—Cœnocytic mycelium. ×900.

E.—Mature zygospore with a well-developed exospore and a large oil globule. ×275.

PLATE 87

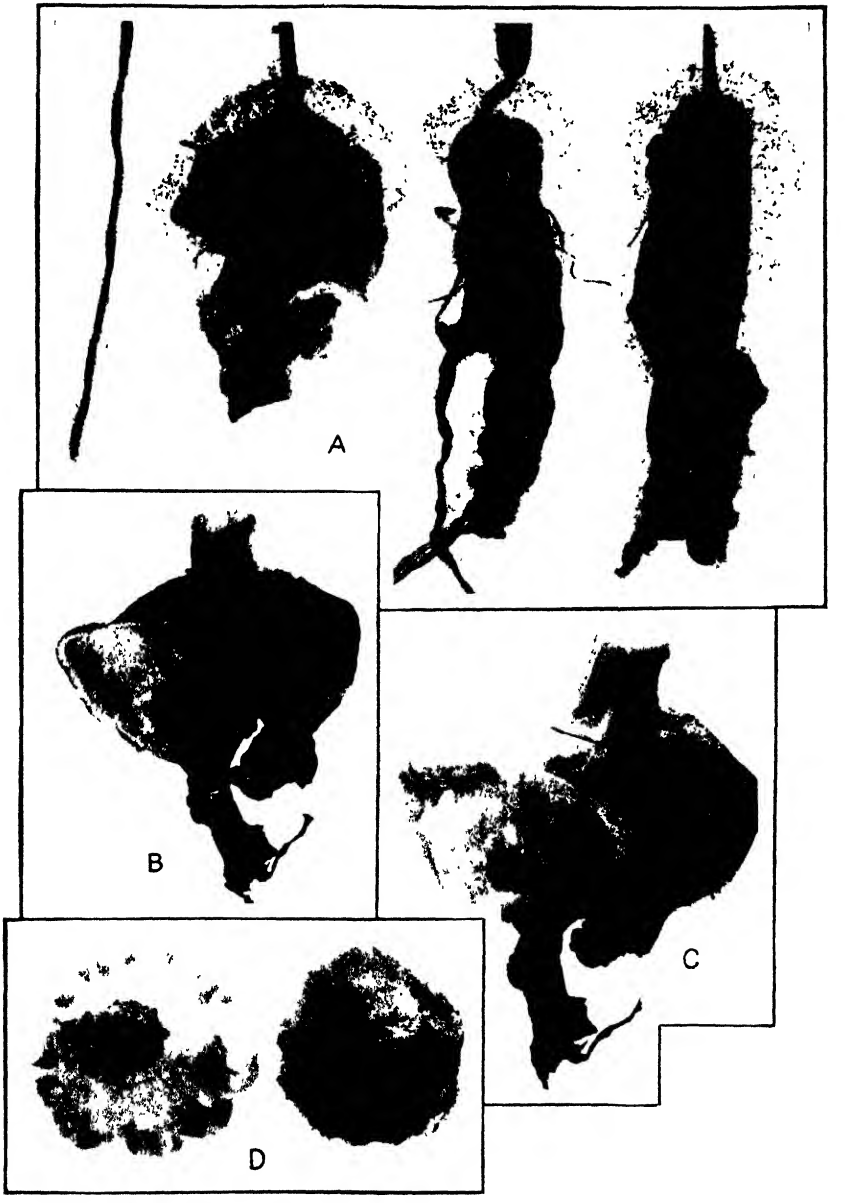
Choanephora cucurbitarum:

A.—Fructifications on squash corollas and flower pedicels. Natural infection.

B.—Natural infection on squash fruit showing the rapidity of the decay when contrasted with figure *C*, which is reproduced from a photograph of the same fruit 15 hours earlier.

C.—A diseased teratological squash.

D.—Infection through the corolla in the fruit at the left and complete decay of the squash at the right.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DISCOVERY OF INTERNAL TELIA PRODUCED BY A SPECIES OF CRONARTIUM

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INTRODUCTION

Adams (1)[1] has recently listed the reports of a number of investigators who have found rust sori developing internally in the host tissue. Both Adams (1) and Wolf (7) fail to mention the work of Bolley and Pritchard (2) and Pritchard (5, 6), who made careful studies of internal sori in wheat grains. A review of the literature shows that pycnia, æcia, uredinia, and telia have been found inside the tissue of the host plant, producing what were apparently normal spores. Table I summarizes all the accounts of internal rust sori which are known to the writer.

TABLE I.—*Summary of accounts of internal rust sori*

Author.	Parasite.	Spore stages.	Host.	Where located.
Adams, J. F. (1)...	*Nigredo caryophyllina* (Schrank) Arthur = *Uromyces caryophilinus* (Schroeter).	II	*Dianthus caryophillus* L.	In leaves.
Atkinson, G. F., and Edgerton, C. W. and Reddick, Donald (7).	*Nigredo caladdii* (Schw.) Arthur = *Uromyces caladdii* Farlow.	I	*Peltandra virginicia* (L) Kunth.	(?)
Bolley, Henry L., and Pritchard, F. J. (2).	*Puccinia graminis tritici* E. and H.	II and III.	*Triticum* sp.....	Beneath bran layer and about embryo of seed.
Eriksson, J., and Henning, E. (5)	*Puccinia glumarum* (Schmidt) Eriks. and Henn.	II and III.	Cereal grains....	In pericarp.
Fromme, Fred D. (4).	*Puccinia claytoniata* Peck.	o	*Claytonia virginica* L.	Deep in parenchyma of stem.
Pritchard, F. J. (6).	*Puccinia graminis tritici* E. and H.	III	*Triticum* sp.....	In seed near hilum.
Pritchard, F. J. (5).	*Puccinia graminis tritici* E. and H.	III	.....do.....	In pericarp of wheat grain.
Reddick, Donald (7).	*Puccinia graminis* Pers.	I	*Berberis vulgaris* L.	In fruit.
Do.....	*Dicaeoma poculiforme* (Jacq.) Kuntze = *Puccinia graminis* Pers.	II	*Secale* sp.....	In stem.
Smith, W. G. (5)...	*Puccinia graminis* Pers.	III	*Avena* sp.....	Next to gluten layer of seed.
Wolf, F. A. (7).....	*Puccinia angustata* Peck.	I	*Lycopus virginicus* L.	In pith and parenchyma tissue of petioles and stems.

[1] Reference is made by number to "Literature cited," p. 332.

A glance at the table shows that practically any part of the host plant may contain internal sori. According to the accounts of different authors, sori were found buried deep in the tissue of the host plants, freeing their spores into hollow stems, or intercellular spaces, and in some cases making room for the lengthening spore chains by forcing aside the surrounding parenchyma cells (Pl. 88, *B*). Of course, the sori in seeds had little room to expand. Fromme (3, 4) has reported odd cases where the pycnium was located in the æcium. The discovery of these internal sori has probably been accidental in all cases, although the investigators may have been looking for abnormal conditions.

OBSERVATIONS ON THE INTERNAL TELIA OF CRONARTIUM RIBICOLA

It will be noted that the fungus genera mentioned in Table I all belong to the Puccinia and Uromyces groups. So far as the writer is aware, there have been no reports of internal sori of any species of the genus Cronartium. In the course of intensive investigations on *Cronartium ribicola* Fisher, the white-pine blister rust, a striking case of the development of internal telia was discovered in the petiole of *Ribes roezli* (Regel) Coville and Britton. The plant was inoculated in July, 1915, and was kept in the greenhouse through the following winter. On February 28, 1916, telia were found by Spaulding [1] on the petiole of one dead leaf and of one partially dead leaf, both still remaining on the plant. These petioles were killed, embedded in paraffin, and sectioned. Close to the base of the leaf the pith and pericycle regions (Pl. 88, *A*) of the petiole were found to be practically stuffed with the mycelium of the parasite, while farther from the leaf base there was less complete destruction of the host tissue. Haustoria, which were larger and more numerous than those in the leaf, suggested that the mycelium was more active in the petiole than in the leaf. At intervals in the tissue of the regions mentioned telia had begun to develop (Pl. 88, *E*). The direction of growth of what would normally be telial columns was very variable, some growing toward the outside, some toward the center of the petiole. Quite naturally the telia could not produce a typical telial column in such cramped quarters, and the variations were such as one would expect; the sori were broadened out (Pl. 88, *D*), the spores compressed, and the columns where partially developed were coiled or bent by the pressure on the free ends (Pl. 88, *C*). Despite the distortion of the columns, the spores quite closely resembled the spores from a normal sorus, lacking, however, in many cases, the heavier brown wall, characteristic of the fully developed spores of an external column. Possibly the walls would have taken on the brown color as the spores matured. In several of the sori, older than the others, the spores were just begin-

[1] Dr. Perley Spaulding, of the Office of Forest Pathology, supplied the material for this study, and kindly furnished the data on the inoculation.

ning to develop the wall thickening and color change. The walls of the spores at the tip of the telial column shown in Plate 88, *C*, were identical in color and thickness with the walls of spores in external columns. The measurements of the internal spores, 10 to 20 by 20 to 40 μ, agreed well with those of normal spores, considering the conditions under which the internal spores were produced. There was at first some doubt as to the true nature of the spores, for some of the sori were surmounted with a peridium like the peridium of a normal uredinium. This would not exclude the possibility that the sorus was a telium, for the normal telial column often arises from an old uredinium. In this case, indeed, there could be no question whether the spores were urediniospores or teliospores, since the mycelium, which, as mentioned above, sometimes stuffed the pith region, was typically binucleate; the spores were uninucleate; and the change from the binucleate condition to the uninucleate condition occurred just after the spores were cut off from the basal cells of the sorus. This cytological evidence, agreeing as it does with cytological conditions in sori producing teliospores, completely established the identity of the internal sori as telia, even when the spores were very young.

The occurrence of the petiole infection is rather common in both wild and cultivated species of Ribes where the general infection is heavy. In two other species examined, *R. nigrum* L. and *R. cynosbati* L., no internal telia had been formed, although the mycelium was abundant and normal telia present on the surface of the petioles. The epidermal region of the petioles of *Ribes* spp. undoubtedly offers more resistance to the developing telia than the epidermis and palisade cells of the leaf. The sori probably are unable to break through the stiffer layer in the petiole; hence, their development as far as possible within the inclosing tissue. Unquestionably internal sori should be regarded as rather common teratological phenomena, developed in spite of their unfavorable position. Morphologically they have no special significance. Their development is to be expected whenever the point at which the sorus begins to form is located beneath a layer of tissue which offers a greater resistance than the developing sorus can overcome. Accidental discovery of the sori after the material had been killed and embedded has precluded any spore germination experiments. Investigators, however, have found the internal mycelium, and the teliospores themselves, to be in a living state after a considerable time, even in comparatively dry tissue. Pritchard (5) figures the teliospores as undergoing a sort of palmella-like division which may be the equivalent of germination. In his material the mycelium was certainly alive for a long time. There is a possibility that the phenomena described by Pritchard (5) might be observed under suitable conditions in the internal teliospores of *Cronartium ribicola*.

SUMMARY

(1) All four types of rust sori have been found developing internally in the host-plant tissue, producing spores which appear quite normal and which fill intercellular spaces or force aside the softer tissue.

(2) The fact that *Cronartium ribicola* Fisher produces internal telia is here reported for the first time. These telia form spores inside the petioles of species of Ribes, chiefly in the pith and pericycle region.

(3) Internal sori should be regarded as rather common teratological phenomena.

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(3) FROMME, F. D.
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(7) WOLF, F. A.
1913. Internal aecia. *In* Mycologia, v. 5, no. 6, p. 303–304, pl. 111.

PLATE 88

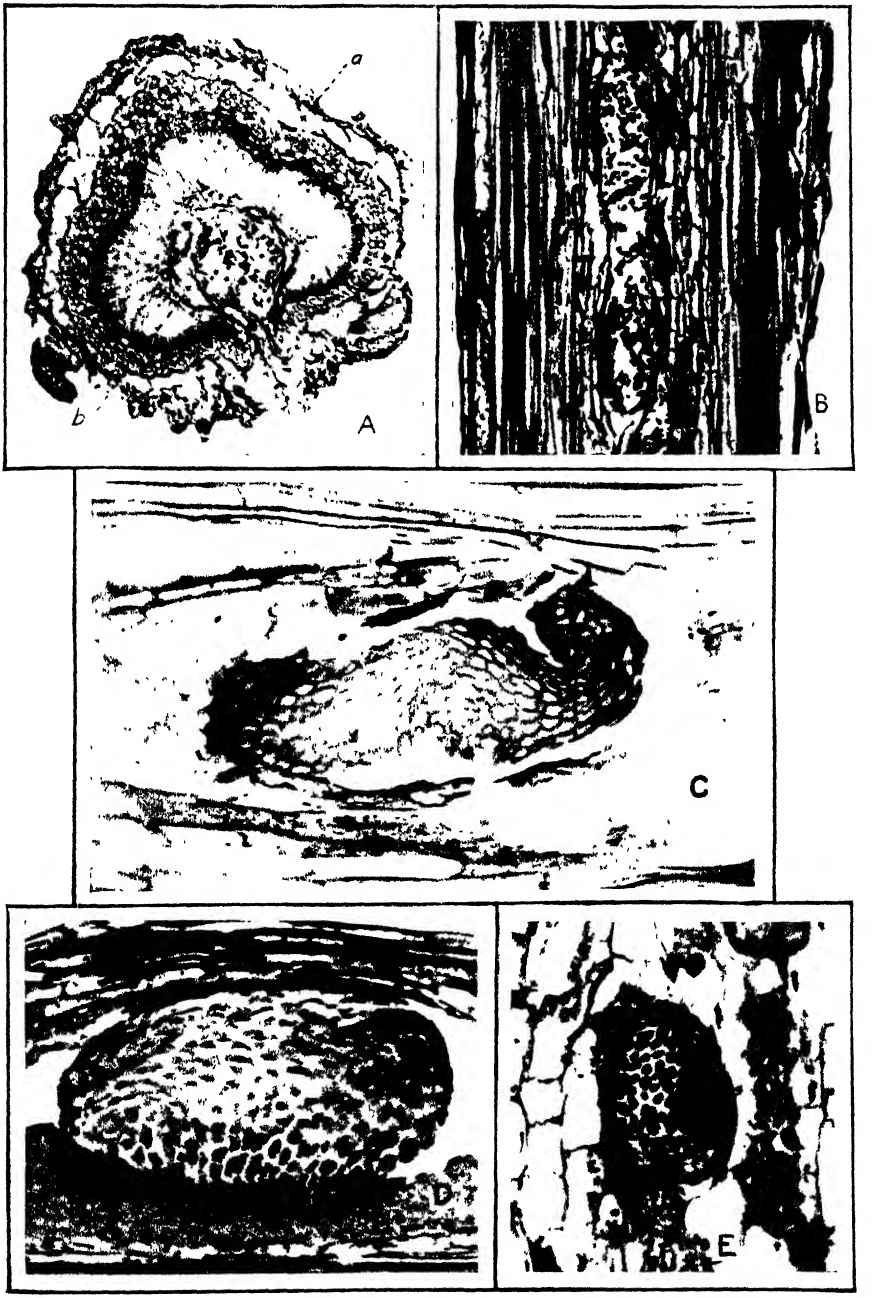
A.—Cross section of a petiole of *Ribes roezli*, showing the pericycle (*a*) and pith (*b*) regions which are stuffed with the mycelium of *Cronartium ribicola*. Most of the pith cells have been destroyed. Teliospores partially fill the cavity.

B.—Longitudinal section of the same petiole, showing the manner in which the sori force aside the parenchyma cells of the pith.

C.—Internal telium of *Cronartium ribicola* from the pericycle region of a petiole of *Ribes roezli*. The telium has been forced to coil on account of the pressure of the surrounding cells.

D.—Internal telium of *Cronartium ribicola* from the pericycle region of a petiole of *Ribes roezli* flattened by pressure.

E.—Young internal telium of *Cronartium ribicola* from the center of the pith region of a petiole of *Ribes roezli*, illustrating the depth at which sori may start to develop.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EFFECT OF IRRIGATION WATER AND MANURE ON THE NITRATES AND TOTAL SOLUBLE SALTS OF THE SOIL

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INTRODUCTION

Plants obtain their food from the soluble salts of the soil; therefore the study of these salts is of great importance to agriculture. There is no method available for measuring exactly what portion of the plant food in the soil can be taken up by crops at any given time; the nearest approach is to extract the soil with some solvent and to determine the quantity of plant food in the solute.

Imperfect as this method is in approximating the available plant food, it yields many valuable data which help to an understanding of some of the complex soil changes.

In the present paper an attempt has been made to determine the effect of varying quantities of soil moisture and manure on the total soluble salts and nitrates that can be extracted by water from the soil. Some of the soils under investigation were kept in the laboratory, others were allowed to stand for long periods in large tanks, while still others were studied under normal field conditions. Comparisons were also made of cropped with uncropped soils in tanks and in the field.

REVIEW OF LITERATURE

Most of the former work on the soluble salts of the soil has been done in humid climates, where conditions are different from those under which the experiments herein reported were conducted. For this reason there is not a complete agreement in the results.

King (8, p. 92–107)[1] found that the ratio of total soluble salts to nitrates varied between 2.14 to 1 and 9.97 to 1 under different conditions. The total soluble salts, and especially the nitrates, were higher in fallow than in cropped soils; and crops used the nitrates faster than they did the other soluble salts.

Investigations by Stewart and Greaves (13, 14) show the application of irrigation water to have a distinct beneficial effect on nitrate formation, being greatest where 15 inches of water were applied. In cropped land there was less nitrogen in the soil during the fall and spring than during the summer. More nitrogen was found in fallow ground in the fall than in the spring, but most of the surplus disappeared during the

[1] Reference is made by number to "Literature cited," p. 359.

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winter. The concentration of the soil solution was higher in fallow soil than in that producing alfalfa, oats, or corn, and nearly always greater in unirrigated than in irrigated soil. It varied widely not only with the crop grown and the amount of water applied but also with depth of soil. There was always a larger amount of nitric nitrogen in fallow plants than in cropped ones, but when the quantity removed by the crop was considered more nitric nitrogen was found in the cropped soil. The amount of nitric nitrogen was more constant in uncropped than in cropped plots and it was found to be comparatively constant in the lower sections of the soil, irrespective of the quantity of water supplied, up to 25 inches.

Contrary to the results of Stewart and Greaves, Russell (12) found that during late summer and early autumn the nitrate content in fallow was higher than in cropped land, even after adding the nitrate taken up by the crop. In a semiarid soil Jensen (4) found no direct relationship between moisture and nitrates. He later (5) found that an irrigation, followed by a heavy rain, evidently diminished the nitrates. More nitrates were found in summer-fallowed than in cropped soil. The average quantity of total nitrogen for the season showed no evident correlation with the quantity of nitrate nitrogen found. Control plots showed slightly more nitrates than those receiving either dry-yard or composted manure, but the reverse was true of the fallow plots.

In a humid climate Weis (17) found no relationship between the percentage of moisture and the nitrate production. King (7) found the same, and also that there is a lack of correlation between nitrates and crop yield.

Watt (16), in South Africa, observed that manure, cultivation, and increased moisture served to raise the nitrate content. That manuring the soil with dung very slightly increased the amount of nitrates was among the observations of Tulaikov (15) in Russia. The depth to which regularly cultivated fallow land was plowed had little influence on the accumulation of nitrates. He also found that the accumulation of nitrates in the soil, under conditions obtaining in the black soil regions of Russia, was much facilitated by the fact that all measures taken to accumulate water in the soil accelerated the accumulation.

McIntyre (11), in reporting the results of 30 years' use of barnyard manure, concluded that soils receiving 6 and 10 tons of manure were practically identical in nitrate content, while the soil that received 8 tons was lowest.

At Cornell University the application of 20 tons of farm manure to the acre to sod on a clay loam soil for three years resulted in a larger production of nitrates on the manured soil than on adjoining unmanured soil.

The work of Johansson (6) showed a close relation between the amount and movement of the soil water and the distribution of nitrates in a heavy clay soil. The nitrates were carried downward by the rain water and

later moved upward as the water was evaporated from the surface of the soil.

For a humid soil Hall (3, p. 230) showed that no more nitrates were leached from a 60-inch lysimeter at Rothamsted than from one 40 inches deep, from which it was concluded that nitrification does not take place below 40 inches from the surface.

Buckman (1) has shown that under dry-farm conditions the formation of nitrates is greater where there is a good supply of soil moisture than where the soil is dry. Intertilled crops after fallow did not seriously deplete the soil for the succeeding grain crop, either in moisture or in nitrates.

Lyon and Bizzell (10), as well as other experimenters, found the formation of nitrates to be influenced by the crop growing in the soil.

At the Rothamsted Experiment Farm (9) nitrates were found to increase, evidently from better moisture conditions, in the fall after a heavy rainfall.

A report of the Washington Agricultural Experiment Station (2, p. 17–18) shows a tendency for the larger quantities of nitrates to be formed in the upper foot of the soil and the smaller quantities deeper down to 36 inches (the lowest depth analyzed); but there were a greater number of high-nitrate samples below 20 inches than above. The highest nitrate content was found in the spring before the plants began to grow.

EXPERIMENTAL WORK

METHODS OF ANALYSIS

In determining the total soluble salts and nitrates 100 gm. of freshly sampled soil were used. To this, 500 c. c. of distilled water were added and stirred thoroughly, then allowed to settle for 20 minutes, after which it was filtered through a Chamberland-Pasteur filter, the first 100 c. c. of the filtrate being discarded.

To get the total salts, 100 c. c. of the filtrate were evaporated to dryness on a steam bath and the residue weighed. The nitrates were determined by the colorimetric method described by Schreiner and Failyer.[1] Both the nitrates and the total salts are expressed in parts per million of dry soil.

SOIL CHANGES IN GLASS JARS

The first experiment was conducted in the laboratory, the object being to study the influence of different quantities of moisture on soil changes under controlled conditions. Glass jars holding 800 gm. of dry soil were made up to percentages of water from 2.95 to 54.47, with differences of about 5 per cent. These were loosely covered with lids to

[1] Schreiner, Oswald, and Failyer, G. H. Colorimetric, turbidity, and titration methods used in soil investigations. U. S. Dept. Agr., Bur. Soils Bul. 31, p. 39–41. 1906.

reduce evaporation, although air could circulate freely. The percentages of moisture were maintained by replacing the water lost at the end of each month. The soil was a sod and was pulverized and sieved before being placed in the jars. An analysis of the soil was made after it had been held under the treatment from January, 1912, to August, 1914.

The results are given in figure 1, which shows the largest amount of total soluble salts in the soil kept at nearly 29 per cent moisture. As the soil moisture increased beyond this amount there was a sharp decrease of soluble salts present.

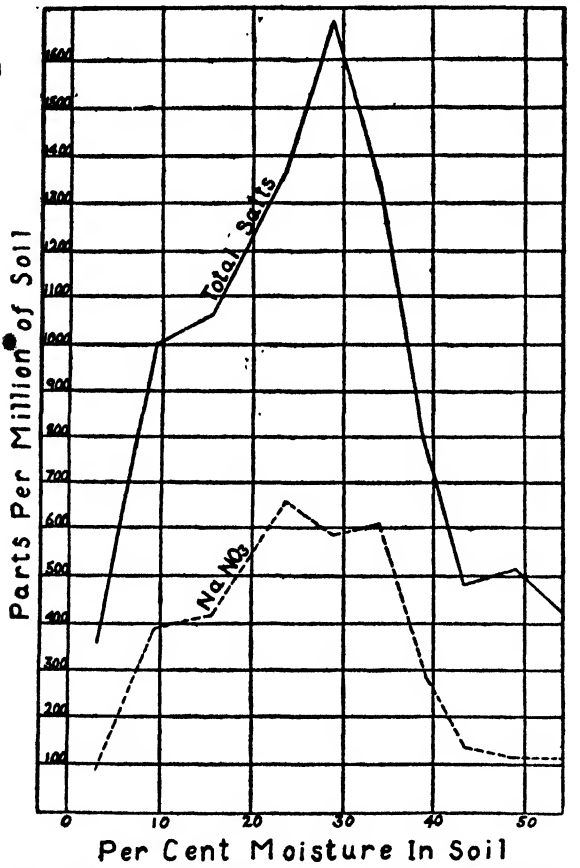

FIG. 1.—Graph showing the total soluble salts and nitrates in soil held in the laboratory, with different quantities of moisture for 2½ years.

The nitrates varied much the same as did the total salts, the main difference being that the maximum quantity of nitrates was found with an initial soil-moisture content of about 24 per cent. It must be remembered that the soil dried out somewhat between waterings; hence, the average moisture would be a little below this amount. The most rapid rise occurred practically at the same places for nitrates and total salts, and the two curves followed each other rather closely. This seems to indicate that the rise in total salts is due largely to the nitrates. Should the nitrates in the soil be considered as $NaNO_3$, the increase, using the lowest percentage as the basis of comparison, will account for nearly half of the increase in total salts. The ratio of total salts to nitrates decreases from 4.34 to 1 at 2.95 per cent moisture to 2.09 to 1 at 23.55 per cent, and then rises to 4.31 to 1 at 48.3 per cent.

These results indicate that in a soil of the nature used in this experiment a moisture content between 23 and 28 per cent is the most advantageous for liberating plant food, especially for the formation of nitrates.

VEGETATION-HOUSE RESULTS

From an experiment begun in 1913 to study the effect of various moisture treatments on the growth of wheat under controlled conditions, samples were taken for the second part of this report. Galvanized-iron tanks, 2½ feet high and 2 feet in diameter, filled with a fertile loam soil containing a high percentage of magnesium and calcium carbonate, were used in this experiment. This is not the same soil as that used in the laboratory study. Enough New Zealand wheat was planted in each tank to make sure of an even distribution of 30 plants in each cropped pot. A half-inch layer of clean sand, covered with paraffined paper, was put over the top of each pot after the plants were up, to reduce water loss by evaporation.

These tanks were placed in groups of six on low cars in an inclosure, 26 by 54 feet, protected on the north and east by board fences 10 feet high. When weighings were to be made, the cars were wheeled beneath a large steelyard and each tank raised from the car by means of hooks attached to the steelyard. The weighings were accurate to 1 pound.

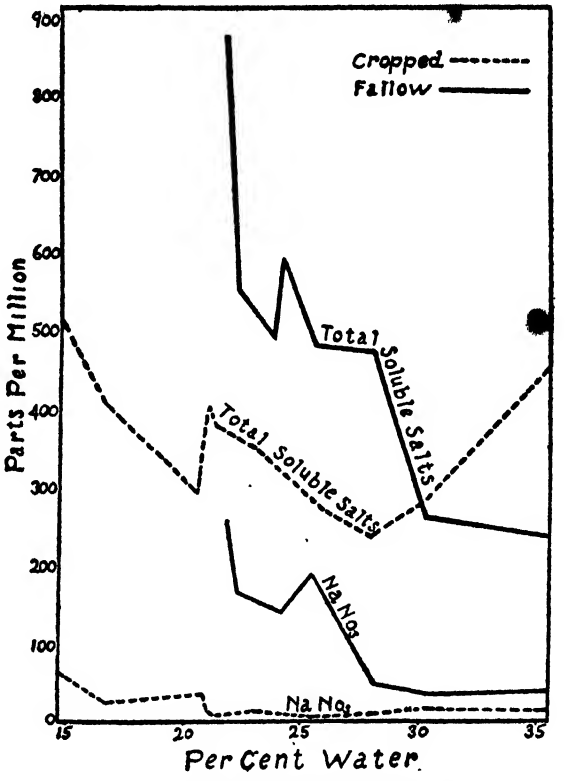

FIG. 2.—Graph showing the total soluble salts and nitrates in cropped and fallow soil maintained at various moisture contents in large tanks.

Twenty tanks were kept at a uniform moisture content throughout the season, one of each treatment being fallow and one cropped each year. Nine other tanks were used, in which the moisture given the plants varied with the stage of growth. The first stage included the period until the plants had five leaves, or until about the last of June; the second from this time until the plants were in the boot just ready to head; and the third from the boot stage to maturity, which usually occurred about the middle of August.

In 1914 and 1915, samples of the soil were taken from each of the tanks after the crop was harvested, and determinations made of total

salts and nitrates to the full depth of the tanks. Each sample was thoroughly mixed before the analysis was made; hence, the figures represent the average for the whole tank. There was a check tank for each treatment.

CONSTANT-MOISTURE CONTENT

The lowest moisture content was a little less than 15 per cent for the cropped and nearly 22 per cent for the fallow tanks, and the highest slightly over 35 per cent for both sets that were maintained at the same moisture content throughout the season.

Figure 2 shows that the lowest moisture content favored the greatest accumulation of total soluble salts and nitrates in both the cropped and

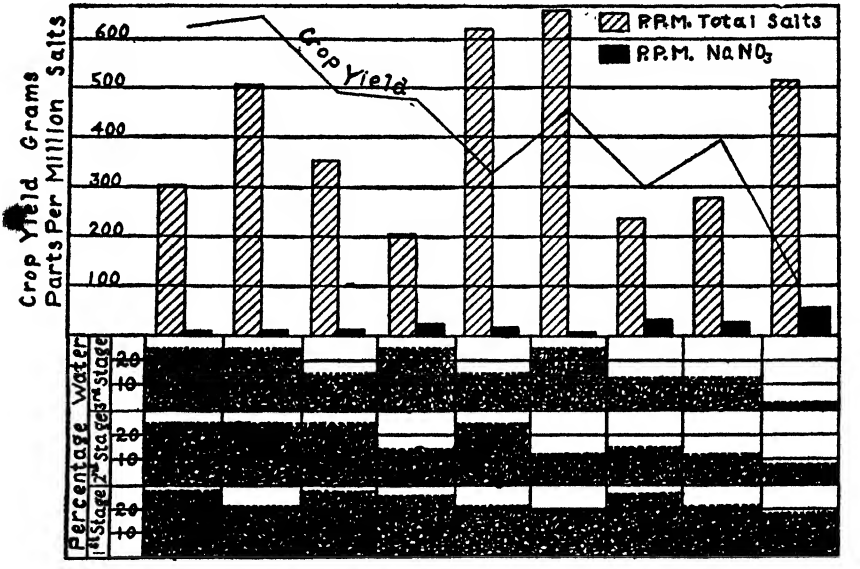

FIG. 3.—Graph showing the total soluble salts, nitrates, and crop yields in tanks containing soil with varying moisture contents during different stages in the growth of the crop.

the uncropped soils. For the cropped tanks containing between 20 and 28 per cent of moisture (where the best crop growth was obtained) the total salts and nitrates, as compared with fallow, are especially low.

The fallow tanks decreased in total salts from 874 parts per million at a moisture content of about 22 per cent to 235 p. p. m. at slightly over 35 per cent. Corresponding to these moistures, the nitrates decreased from 250.9 to 35.8 p. p. m. It will be noted that the ratio of total salts to nitrates is much larger for the cropped than for the fallow plots, showing that the plants take up nitrates faster than they do the other salts.

VARIATION IN MOISTURE AT DIFFERENT STAGES

Figure 3 shows the percentage moisture maintained in the second set of tanks for each stage in the growth of wheat, the total salts and

nitrates found in the soil after the crop had been harvested, and the total yield of grain and straw. The moisture treatments in the illustration have been so arranged that the amount of water in the soil decreases from left to right.

The total salts do not seem to vary consistently with any of the other determinations shown. In general, however, the nitrates vary inversely as the percentage soil moisture and the crop yield, the crop yields standing somewhat in proportion to the total amount of water for the season in the tanks. The variations are thought to be due largely to changes in soil-moisture content at the different stages.

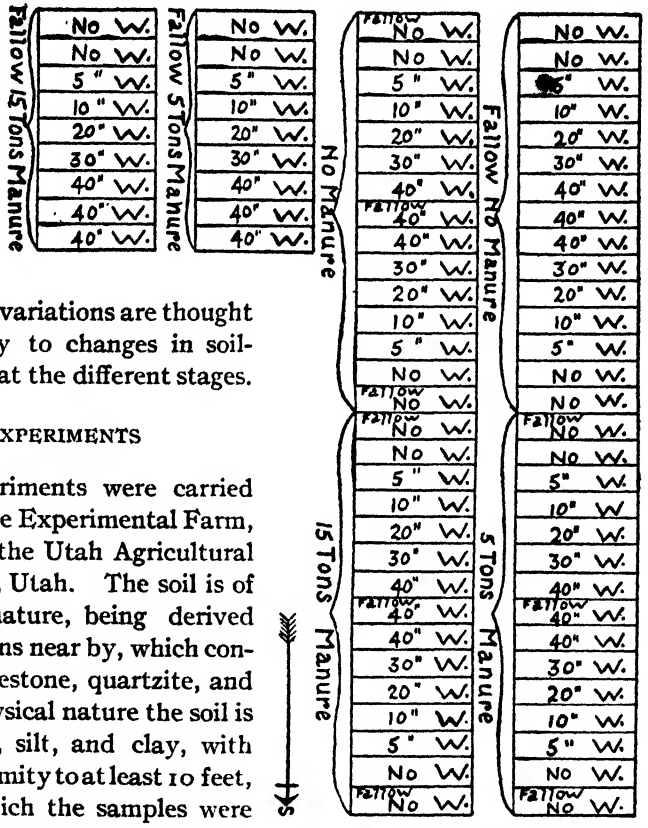

FIG. 4.—Arrangement and treatment of plots in field experiment.

FIELD EXPERIMENTS

The field experiments were carried on at the Greenville Experimental Farm, 2 miles north of the Utah Agricultural College, at Logan, Utah. The soil is of a sedimentary nature, being derived from the mountains near by, which consist largely of limestone, quartzite, and dolomite. In physical nature the soil is mostly fine sand, silt, and clay, with remarkable uniformity to at least 10 feet, the depth to which the samples were taken. The soil contains about 43 per cent of calcium and magnesium carbonates and is rich in all the essential plant-food elements with the exception of nitrogen. The plots had received no manure for many years before the experiment was begun. A more detailed description of the soil and conditions of the farm have been published by the Utah Station.[1]

Figure 4 shows the arrangement of the plots and the treatments received. There were 12 cropped and 18 fallow plots unmanured, 12 cropped and 12 fallow manured at the rate of 5 tons, and 12 cropped and 12 fallow manured at the rate of 15 tons to the acre.

[1] Widtsoe, J. A., and McLaughlin, W. W. The movement of water in irrigated soils. Utah Agr. Exp. Sta. Bul. 115, p. 197–268, illus. 1912.

In each manuring treatment two cropped plots received each of the following quantities of irrigation water: None, 5, 10, 20, 30, and 40 inches. This gave 6 cropped plots for each irrigation treatment. Besides the cropped soils, 6 plots receiving no water, 4 with 40 inches, and 2 of each of the other irrigated, unmanured plots were left fallow. Each of the manured, fallow series contained 4 plots unirrigated and 4 irrigated at the rate of 40 inches, together with one plot for each of the other moisture treatments each year.

The plots were 7 feet wide and 24 feet long, with 4 feet of space between. Two rows of Dakota Sunshine corn were planted on each of the cropped plots, and one row in the spaces between the plots to make the conditions more uniform.

Except for the year 1912, when the weather prevented the usual preparation of the land, the plowing was done in the fall and the soil

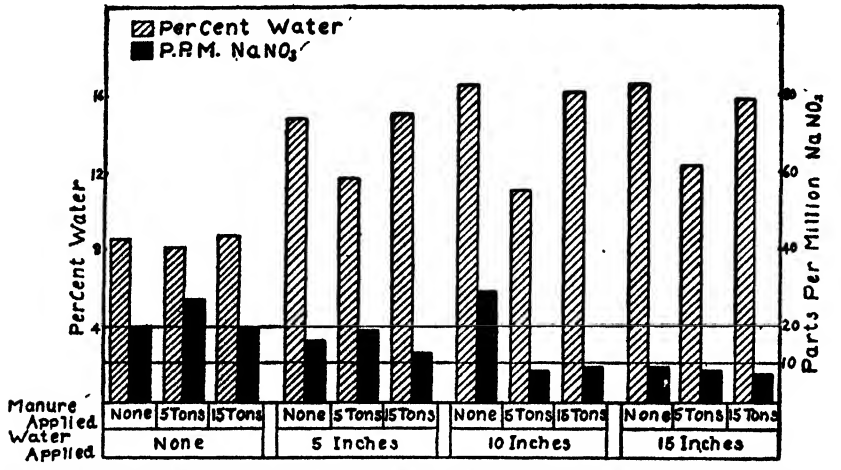

FIG. 5.—Graph showing the soil moisture and nitrates on August 7, 1911, to a depth of 3 feet in cropped soil to which different quantities of water and manure had been added.

left rough until spring, when the manure was added to the plots which received this treatment. Later, the manure (mixed horse and cow manure, fairly well rotted) was thoroughly disked or plowed into the land.

The water was distributed from wooden flumes as follows: For plots receiving 5 inches, 2½ inches each at the beginning of tasseling and at roasting-ear stage; plots receiving 10 inches, 5 inches at each of the above two stages; plots receiving 20 inches, 5 inches each when the plants were 12 inches high, at the beginning of tassel, at bloom, and at roasting-ear stage; plots receiving 30 inches, 5 inches each when plants were 12 inches high, 10 days later, at beginning of tassel, at bloom, at roasting-ear stage, and 10 days later; and plots receiving 40 inches, applications of 5 inches when plants were 12 inches high, and this amount each week thereafter until all water was added. The fallow plots were irrigated at the same time as the cropped.

The experiment was begun in 1911, but only results with the nitrate and moisture content of the soils for the midseason period were taken during that year. No determinations were made in 1912, but midsummer data for 1913 and complete results for the fall seasons of 1913, 1914, and 1915 are included. As the season of the year is thought to influence the production of nitrates to quite an extent and because the water applied, as well as the depths sampled, varied, the two preliminary trials are considered separately.

Preliminary Tests

Summer of 1911

The samples taken on August 7, 1911, include only the first 3 feet of soil at a time when the most water applied on any plot was 15 inches.

Figures 5 and 6 give the average moisture and nitrates to a depth of 3 feet, figure 5 representing the cropped and figure 6 the uncropped plots.

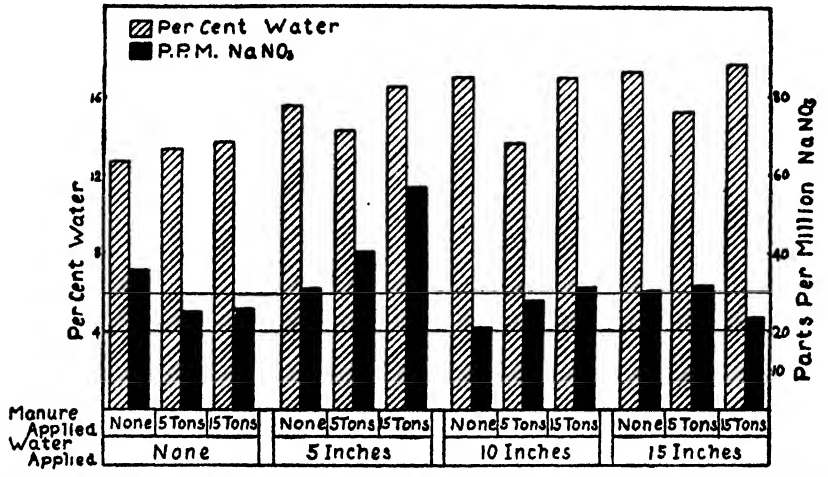

Fig. 6.—Graph showing the soil moisture and nitrates on August 7, 1911, to a depth of 3 feet in fallow soil to which different quantities of water and manure had been applied.

On an average the cropped plots receiving no manure contained more water than those receiving either 5 or 15 tons, and the difference is most noticeable with the larger applications of irrigation water. In depth the first foot of the 15-ton-of-manure plots contained more water than those with no manure, and the first foot of the 5-ton plots proportionately more than the other feet when compared with the unmanured. For these 3 feet the moisture increased with the depth of soil. The land receiving manure at the rate of 5 tons contained an average of about 3 per cent less water than that receiving none or 15 tons, and was more than this for the larger quantities of water applied.

While the nitrates in the cropped plots to which 5 tons of manure were added were higher than those receiving none, where no water and 5 inches of water had been applied, the average for the unmanured plots was higher than that of either of the manurial treatment soils. The third foot of the three treatments differed little.

In general, the nitrates in the first 3 feet vary inversely with the water applied.

The moisture content of the fallow plots receiving manure at the rate of 15 tons to the acre averages highest, the unmanured slightly lower, mainly in the third foot, and those on which 5 tons were applied considerably below the two others, especially for the larger irrigations.

With 15 tons of manure the average nitrate content of the soil was highest, and with no manure lowest, these differences occurring mostly with 5 and 10 inches of water. The nitrates of the manured plots show a rather marked rise when a 5-inch application of water had been used, while more than this amount causes a decrease. A tendency for a steady decrease of nitrates with increasing percentages of water was not noticed in the fallow plots as it was in the cropped.

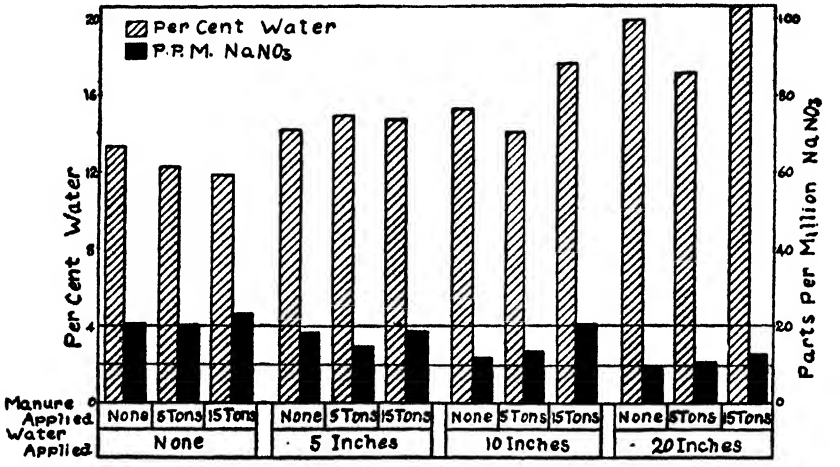

FIG. 7.—Graph showing the soil moisture and nitrates on July 21, 1913, to a depth of 10 feet in cropped soil to which different quantities of water and manure had been added.

Comparing cropped and fallow plots, we find that comparatively more of the nitrates are used by the plants on the 15-ton-of-manure soil than on the 5-ton, and more on the 5-ton than on the unmanured. For the 3 feet shown the difference in nitrates between fallow and cropped soil decreases with increasing depth. Except for the unirrigated soil the nitrates, as shown at this part of the season, are influenced much more by the crop than is the percentage water. The cropped soil contained on the average approximately 15.3 p. p. m. of nitrates, expressed as sodium nitrate ($NaNO_3$), and the fallow a little more than double this quantity.

SUMMER OF 1913

On July 21, 1913, the plots were again sampled, this time to a depth of 10 feet. The moisture and nitrates for all cropped plots are shown in figure 7, and for all fallow plots in figure 8. These results show that 5

tons of manure reduced the moisture content of the cropped soil, and only slightly affected the nitrates, while 15 tons increased both the moisture and nitrates, especially for the larger applications of water.

For 5 and 10 inches of irrigation water, when averaged for all manurings, the increase in moisture content due to irrigation was more rapid in the top few feet than below; but for 20 inches the increase was rather uniform throughout the entire 10 feet. Although the nitrates were proportionately higher in the unirrigated plots at medium depths, the decrease in nitrates for increasing amounts of water was shown more in the top few feet than at the greater depths.

In the fallow plots the highest percentage of moisture, on an average, was found under a manuring of 15 tons, and the least with no manure, but the differences were not great. The nitrates were also lower in the

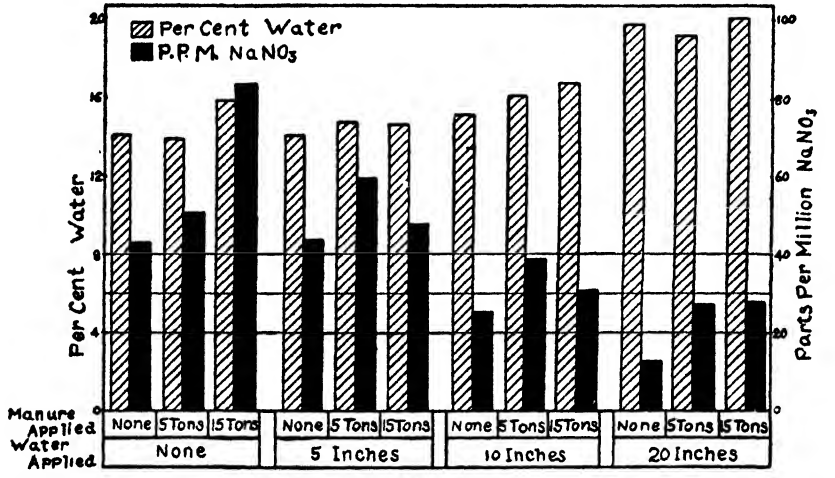

FIG. 8.—Graph showing the soil moisture and nitrates on July 21, 1913, to a depth of 10 feet in fallow soil to which different quantities of water and manure had been added.

unmanured than in the manured land. The larger manuring was usually the more beneficial to the production of nitrates, especially in the unirrigated land, although the 5-ton-per-acre plots contained somewhat larger amounts for 5 and 10 inches of water applied than the 15. The decrease in nitrates for increasing irrigations was rather marked.

At this stage of growth practically the only difference in the moisture content between cropped and fallow plots is the greater loss on the cropped plots receiving no water, especially on the manured land. In a general way the nitrates of both cropped and fallow soils decrease with increasing amounts of irrigation water. The fallow plots show the effect of the increasing quantities of water more than do the cropped, and the influence is felt to greater depths in the fallow soils.

The effect of cropping on nitrates was much greater for soils receiving manure, and especially where the amount of irrigation water applied

was small. This last is most notable in the first and again in the middle to lower depths of sampling. The cropped plots averaged 16.4 p. p. m. and the fallow 40.6 p. p. m. of sodium nitrate.

AVERAGES FOR THE FALL SEASONS OF 1913, 1914, AND 1915

TOTAL SOLUBLE SALTS IN CROPPED SOIL

From Table I and figure 9, which show the total soluble salts found in the cropped plots to a depth of 10 feet about the first of September, just after the corn crop had been removed, we find that with a slight exception on the lands receiving 10 inches of water, manuring with 5 tons per acre resulted in a lower salt content than with no manure. This difference is most marked with no and 5 inches of water applied,

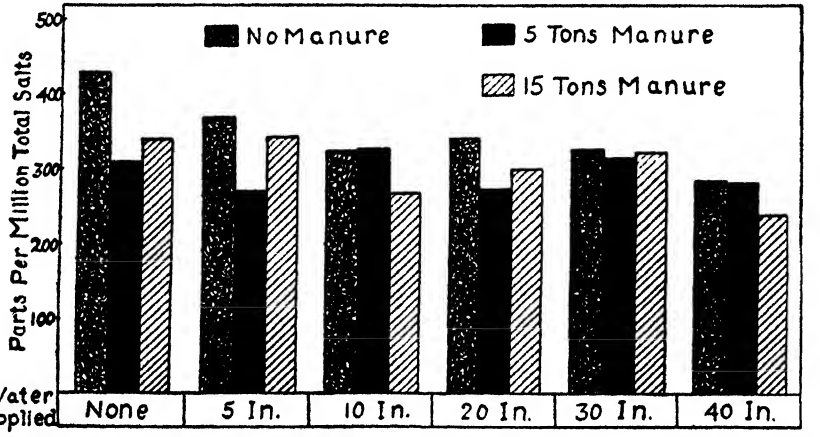

FIG. 9.—Graph showing the total soluble salts in the fall in cropped soil receiving different quantities of manure and irrigation water. Average of 3 years to a depth of 10 feet.

and with 10, 30, and 40 inches is hardly noticeable. In depth, although there are quite a number of exceptions for the individual feet in the irrigation treatments, the average shows a difference in total salts favoring no manure. The greatest variation is at the fifth, sixth, and seventh feet, and least at the first and ninth feet.

Plots receiving 15 tons of manure showed less total salts for every irrigation treatment than those receiving none. The lower feet of the plots receiving these two treatments show more variation than the upper ones.

It is mainly for no water and 5 inches, and the first, second, third, and sixth feet in depth that the averages for the 15-ton plots exceed the 5-ton plots.

The salts of the cropped soil receiving no manure tend to vary inversely with the water applied; with 5 tons practically no regular variation is shown, while 15 tons indicates an irregular variation inversely with the water applied. The average for all manuring treatments,

therefore, shows a tendency for an inverse variation, the difference between the plots receiving no water and those receiving 40 inches being 90 parts per million.

TABLE I.—*Total soluble salts (in parts per million) in the fall at various depths in cropped and fallow soil receiving different quantities of manure and irrigation water. Average for three years*

CROPPED SOIL

Depth of soil.	Soluble salts (p. p. m.).																			
	No water.			5 inches of water.			10 inches of water.			20 inches of water.			30 inches of water.			40 inches of water.			Average.
Manure applied (tons)	0	5	15	0	5	15	0	5	15	0	5	15	0	5	15	0	5	15	
Feet.																			
1	341	304	322	372	288	404	361	372	267	335	234	393	291	300	389	280	314	270	324
2	517	325	388	436	318	385	386	337	299	347	228	360	308	402	375	274	238	228	342
3	506	319	351	271	283	377	412	386	302	375	298	355	284	275	436	266	356	288	341
4	435	416	380	425	259	344	344	303	363	354	309	298	317	300	235	244	271	263	326
5	477	283	310	261	226	299	324	287	273	358	260	252	369	282	244	223	218	205	286
6	374	247	304	294	260	359	256	253	237	345	300	249	386	286	338	268	278	227	292
7	514	278	343	361	238	285	287	348	211	355	232	258	325	322	358	365	277	183	308
8	365	353	360	487	267	359	322	342	278	296	282	296	318	284	306	314	333	238	322
9	428	294	347	318	314	314	303	357	198	335	304	273	365	361	234	299	262	255	309
10	348	271	301	390	247	324	249	296	252	306	279	254	302	314	269	322	290	243	292
Average	431	309	340	361	270	345	324	328	268	341	273	298	326	313	322	285	284	240	314

FALLOW SOIL

1	455	420	317	422	306	532	469	517	319	440	577	331	487	710	405	284	442	266	428
2	382	465	437	365	320	443	381	425	321	518	341	318	536	519	365	324	501	222	399
3	285	393	430	412	454	407	381	428	316	452	420	328	403	396	372	315	353	271	379
4	484	383	474	444	324	448	374	483	454	366	456	289	460	397	326	292	253	229	385
5	356	349	451	427	324	411	364	366	306	357	444	372	361	332	480	304	580	282	381
6	461	360	339	389	362	368	397	402	306	435	511	446	354	422	401	297	300	229	377
7	335	353	360	362	296	341	397	347	282	388	389	396	359	367	444	334	257	210	345
8	355	289	398	322	281	323	362	425	284	316	429	319	384	391	311	322	301	320	341
9	396	427	287	442	324	304	278	346	273	342	250	302	362	313	447	299	299	352	336
10	335	265	306	433	266	290	266	316	347	353	393	272	378	339	375	290	307	281	323
Average	385	370	380	402	326	387	367	405	321	397	421	337	409	419	393	306	359	266	369

In general, it will be noticed that the plots receiving no water contained more total salts in every foot, except the first, than the other treatments. When more than 10 inches of water were applied, the salt content of the top foot of the unirrigated soil was practically equal to or greater than the same foot of those receiving water. The top 2 and the lower 3 feet of the plots receiving 5 inches of water contain more salts than the corresponding feet of the other irrigated plots. When 20 inches were applied, some of the salts seem to have moved below 10 feet; the 30-inch irrigation shows this tendency still more, while the 40-inch application seems to have driven much of the salts below the depths of sampling, since every foot of this treatment is somewhat below the others. Especially is this true for the first 5 feet. In general, the first 4 feet of the first four irrigation treatments are noticeably higher than the feet below, while for the last two irrigations the differences are somewhat more evenly distributed throughout for depth.

TOTAL SOLUBLE SALTS IN FALLOW SOIL

As shown in Table I and figure 10, the total salt content of the fallow soil receiving 5 tons of manure was greater with irrigations of 10 inches or more than where none was applied; but with no or only 5 inches of water the salt content was higher where no manure was added. In most of the upper feet the average salts for the 5-ton-of-manure plots exceeded that with no manure, but the differences are in most cases not very striking.

For every moisture treatment the salt content of the plots receiving 15 tons of manure was lower than where no manure was added, but the difference is not marked where no water was applied. The fifth foot is the only depth at which the salt content of the 15-ton application ex-

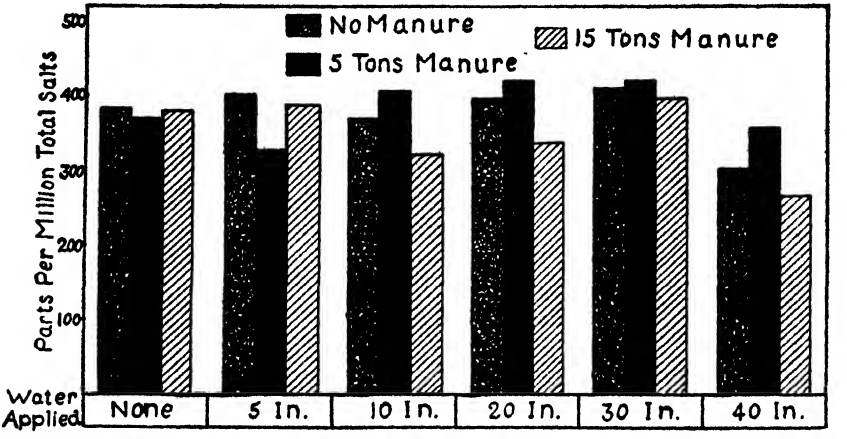

FIG. 10.—Graph showing the total soluble salts in the fall in fallow soil receiving different quantities of manure and irrigation water. Average of 3 years to a depth of 10 feet.

ceeded that of no manure, and the upper feet differed more than the lower.

With no water and 5 inches of water, especially with the latter, the total salts under the plots receiving 15 tons of manure exceeded those with 5 tons, but the other irrigation treatments show quite a wide margin (26 to 93 p. p. m.) in favor of the 5 tons of manure. With regard to depth, the difference in favor of the 5-ton application is mainly in the first 3 feet, the approach being quite gradual from a difference of 133 p. p. m. at the first to 13 at the fourth foot.

The average total salt content of plots receiving 30 inches of water is higher than for the other treatments, especially for the first and second foot in depth. In every foot except the fifth the 40-inch application resulted in a much lower salt content than did the other treatments, and in this exception the difference is not great.

The tendency for the salts to decrease with depth is much more marked in the fallow plots than in the cropped, although with no water and 40 inches for the fallow soil the tendency is hardly noticeable.

As an average the difference in total salt content between fallow and cropped soils was least noticeable on the unirrigated plots and increased with the water applied up to 30 inches. Only 41 p. p. m. more salts were in the fallow than in the cropped soil for an application of 40 inches of irrigation water. The fallow plots differ from the cropped more where the manuring was 5 tons to the acre than for the other treatments. The difference is also more pronounced for the top feet than for the lower ones with applications of no manure and 5 tons, although for 15 tons the lower feet of the two treatments differ most.

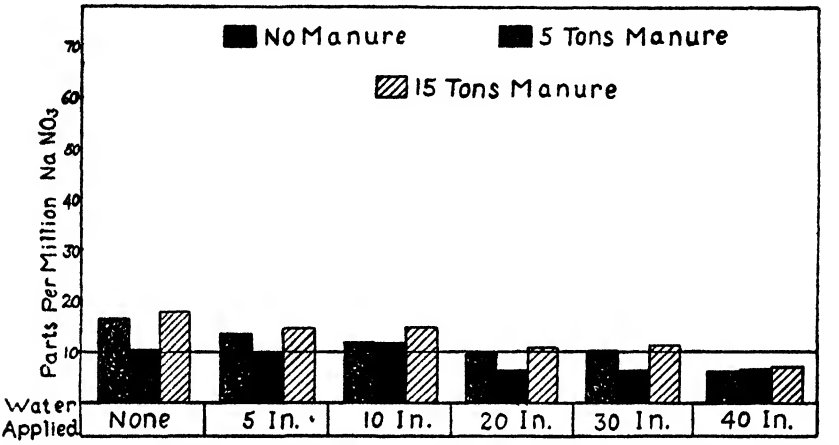

FIG. 11.—Graph showing the nitrates in the fall in cropped soil receiving different quantities of manure and irrigation water. Average of 3 years to a depth of 10 feet.

For most irrigation treatments the total soluble salts in the top foot of the cropped and fallow soils differed considerably more than the average for all feet. This is much more noticeable for the plots receiving 30 inches than for the others both in the first and second foot.

Tables II and III summarize the effects on soluble salts of manure and moisture separately in both cropped and fallow soil.

NITRATES IN CROPPED SOIL

The nitrates, expressed as sodium nitrate in parts per million of dry soil, found in the same plots as discussed above, are shown in Table IV and figures 11 and 12.

Only when the application of water was 40 inches did the nitrates of the cropped plots receiving 5 tons of manure exceed those of unmanured plots, and in this case the difference was small. The greatest variation between the two was for no water. Although the greatest average

difference in nitrates was only about 6 p. p. m., in favor of the unmanured soil, all but the tenth foot in depth show a variation in the same direction.

Manuring with 15 tons to the acre resulted in the formation of over 1.6 p. p. m. more nitrates than where no manure was applied. On the average, the first and third feet in depth are the only ones that this manuring caused to differ noticeably in nitrates.

TABLE II.—*Effect of manure on the total soluble salts in cropped and fallow soil. Average of all moisture treatments for three years*

Depth of soil.	Soluble salts (p. p. m.).							
	No manure.		5 tons of manure.		15 tons of manure.		Average.	
	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.
Feet.								
1	330	426	302	495	341	362	324	428
2	378	418	308	428	339	351	342	399
3	352	375	319	408	351	354	341	379
4	353	403	310	383	313	370	325	385
5	336	358	260	399	263	384	286	380
6	321	389	271	393	286	348	292	377
7	368	362	282	335	273	339	308	345
8	347	344	310	353	306	326	321	342
9	341	354	315	327	276	328	311	336
10	319	343	280	314	274	312	291	323
Average	345	377	296	384	302	347	314	369

TABLE III.—*Effect of irrigation water on the total soluble salts in cropped and uncropped soil. Average of all manure treatments for three years*

Depth of soil.	Soluble salts (p. p. m.).													
	No water.		5 inches of water.		10 inches of water.		20 inches of water.		30 inches of water.		40 inches of water.		Average.	
	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.
Feet.														
1	322	397	355	420	334	435	321	449	327	534	288	331	325	428
2	410	428	380	376	341	376	312	392	362	473	247	349	342	399
3	392	369	310	424	367	386	342	400	332	390	303	313	341	380
4	410	447	343	405	336	437	320	370	284	395	248	258	324	385
5	356	385	262	387	294	346	290	391	298	391	216	388	285	381
6	308	387	305	373	249	369	298	464	337	392	258	275	293	377
7	378	349	295	333	281	342	280	390	335	390	275	267	307	345
8	349	347	371	309	314	357	297	355	303	362	295	314	321	341
9	367	370	349	357	286	299	304	298	331	374	272	317	318	336
10	307	302	320	330	266	310	280	339	295	375	285	293	292	325
Average	360	378	325	371	307	363	304	385	320	407	270	311	314	369

Without consideration of the effect of the crop and comparison with the unmanured soil, the larger amount of manure affected the nitrates proportionately less than did the smaller. The difference between the two treatments favoring 15 tons of manure varied somewhat inversely

with the amount of water applied, the difference being 7.1 p. p. m. with no water and 1.0 p. p. m. with the 40-inch irrigation. In the upper feet the greater accumulation of nitrates under the larger manuring is more clearly evident than in the lower feet, although in almost every foot the difference is in favor of the 15-ton application.

TABLE IV.—*Total nitrates in the fall at various depths in cropped and fallow soil receiving different quantities of manure and irrigation water. Average for three years*

[Results expressed as p. p. m. of sodium nitrate]

CROPPED SOIL

Depth of soil	Total nitrates.																					Average.
	No water.			5 inches of water.			10 inches of water.			20 inches of water.			30 inches of water.			40 inches of water.					
Manure applied (tons)..... ...	0	5	15	0	5	15	0	5	15	0	5	15	0	5	15	0	5	15			
Feet.																						
1.. .	18.8	12.7	30.9	19.4	12.1	20.0	17.0	13.3	26.1	12.1	14.5	17.6	11.5	16.4	23.6	9.1	10.3	12.7	16.6			
2. .	17.6	8.5	23.6	13.9	7.9	12.1	15.2	10.3	14.5	12.1	6.1	10.3	8.5	7.9	13 3	10.9	3.6	12.7	11.6			
3.	14.5	7.9	15.2	9.1	7.3	20.6	9.7	12.7	15.8	7.3	6.7	9.1	7.9	6.1	8.5	4.2	3.6	5.5	9.5			
4. .	21.8	7.3	20.6	13.9	7.9	17.0	12 1	14.5	18.8	9.1	6.7	10.3	10.9	4.2	7.9	4.8	4.8	3.0	10.9			
5	19.4	9.7	22.4	13.3	10.3	9.1	8.5	7.9	13.3	7.9	4.2	11.5	7.9	3.6	5.5	4.8	4.8	12.1	9.8			
6..	18 2	15.8	13.3	12.1	9.7	9.7	8.5	6.7	11.5	9.1	3.6	11.5	10.9	4.2	6.1	6.7	11.5	2.4	9.5			
7...	15.2	10 3	13.3	12.7	5.5	15.7	9.1	10.3	10.3	12.1	6.1	10.1	13 3	5.5	10.9	7.3	9.1	12.1	10.5			
8..	17.6	12.1	12.1	10.3	12.1	16.4	9.7	10.9	10.3	10 9	7.9	9.7	12.7	5.5	12.7	7.9	9.1	2.4	10.6			
9...	11.5	8.5	15.2	14.5	10.9	10.3	8.5	12.1	11.5	8.5	6.7	9.1	11.5	6.1	10 9	6 1	6.7	10.3	9.9			
10	6 7	12 1	9.1	9.1	9.7	14.5	13.9	12.7	15.2	10 1	6.7	9.1	6.7	7.9	11.5	4.8	6.1	6.7	9.6			
Average .	16.1	10.5	17.6	12.8	9.3	14.5	11.2	11.1	14.7	9.9	6.9	10.8	10.2	6.7	11.1	6.7	7.0	8.0	10.8			

FALLOW SOIL

1	46.7	32.7	89.1	43.6	40.0	107.3	23.6	76.4	44.2	23.6	35.8	30.3	15.8	34.5	43.6	13.3	27.9	26.1	41.9			
2	28.5	27.3	76.3	31.5	27.3	96.4	19.4	64.8	34.5	17.6	36.4	33.9	13.9	37.6	32.7	12.7	18.8	25.5	35.3			
3	36 4	32.1	51.5	30.9	35.8	59.5	16.4	58.2	30.4	15.8	35.2	24.8	17 0	42.4	33.9	20.0	19.4	23.6	32.7			
4	46.1	29.7	47.3	39.4	22.4	61.2	22.4	64.2	33.9	23.0	36.4	35.8	15.2	35.8	25.5	11.5	18.8	20.6	32.7			
5	40.6	32.7	63.6	33.9	33.3	44.8	27.9	41.8	29.1	31.5	41 8	43.6	14.5	23.0	23.6	12.1	24.2	11.0	31.8			
6	39 4	29.1	66.6	37.6	30.9	71.5	32.1	49.7	32.7	30.3	43.0	41.2	14.6	38.2	34 5	15.8	24.2	18.2	36.1			
7	26.7	32.7	81.8	28.5	29.7	55.8	28.5	38.8	29.7	19.4	39 4	38.2	14.5	38.8	37.0	10.4	20.0	17.6	33.0			
8	29.7	23 0	62.4	24.2	23.0	52.7	24.2	31.5	27.3	24 8	24.2	34.0	11.5	27.3	31 0	14.6	28.5	24.2	28.8			
9.	23.6	18.2	44.8	13.9	32.7	40.0	23.0	24.2	24.2	24.2	21.2	37.0	12.1	26.7	35.8	12.7	20.6	25.5	25.6			
10 . .	20.6	27.3	64.2	20.0	38.2	61.8	23.6	34.5	27 9	23 0	24.2	32.7	16.4	27.9	31.5	14.0	17.6	21.2	29.3			
Average	33 8	28.5	64.8	30.4	31.3	65 1	24.1	48.4	32.0	23.3	33.8	35.2	14.6	33.2	32.9	14.3	22.0	21.4	32.7			

The tendency for the nitrates to vary inversely with the irrigation water applied is noticed in figure 11 to be more pronounced for no manure and 15-ton applications than for 5-ton applications, but all show a little inclination in this direction. The effect of the irrigation is also seen at almost every foot in depth. As an average, the first two and the middle feet of the unirrigated plots are proportionately higher in nitrates than the other feet when compared with other moisture treatments. The tenth foot of the unirrigated soil is low, in some cases being lower than the plots with water applied. Of the irrigated plots, the top 4 feet of those plots receiving 10 inches and the top foot of those receiving 30 inches of water are high.

NITRATES IN FALLOW SOIL

A consideration of the nitrates found in fallow land, as given in Table IV and figure 12, will show that, except for the plots receiving no water, larger amounts were found in the soils on which an application of 5 tons of manure was made than where no manure was added. With 10 inches of water as an irrigation, the nitrates in the plots receiving 5 tons of manure were double those in the unmanured plots, and more than double with 30 inches of irrigation water. Although the nitrates in the soils receiving no manure and 5 tons differ quite uniformly with regard to depth, each of the first 3 feet of the manured plots contained proportionately more than the unmanured.

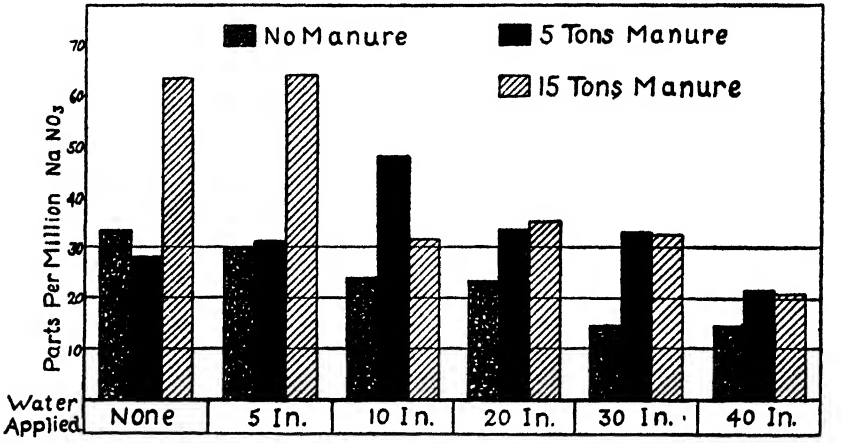

FIG. 12.—Graph showing the nitrates in the fall in fallow soil receiving different quantities of manure and irrigation water. Average of 3 years to a depth of 10 feet.

The soil receiving 15 tons of manure contained, on the average, 5 p. p. m. of nitrates more than did that receiving no manure. This difference was greater for the plots receiving 0 and 5 inches of water than for those receiving more water, and also greater in the upper 2 feet than below this depth.

With no water and with irrigations of 5 inches, the plots on which 15 tons of manure were applied each contained more than twice as high nitrates as did the plots with 5 tons, although with 10, 30, and 40 inches of water the 5-ton applications were better than the 15-ton. The average differences in nitrates between these treatments are a little greater in the first 2 feet than deeper, although the lower 5 feet also show quite a pronounced influence.

As with the cropped plots receiving no manure and 15 tons, the fallow indicates a tendency for the nitrates to decrease with increasing applications of water, while the land receiving 5 tons of manure shows an increase in nitrates until 10 inches of water are applied before the de-

crease began. The average for all manures on fallow land shows that the nitrates began to decrease in amount when the irrigation was 10 inches. The first 5 inches of water applied resulted in more nitrates for the first 3 feet than where no water was applied. The differences in favor of the smaller irrigations appear more in the upper feet than in the lower in most cases.

It may be seen that although the nitrate content of the fallow soils is more than double that of the cropped for no manuring and more than three times for the respective treatments when manure was added, the general relationship between the cropped and fallow plots for nitrates is somewhat similar for the different manuring treatments. This is found both for the water applied and the depth, although the fallow plots are inclined to be more irregular than the cropped ones. The difference between cropped and fallow soils is less pronounced with the larger irrigations than with none or the smaller ones. The proportionate increase in nitrates for increasing amounts of manure is greater for the fallow than for the cropped soils. Fallow and cropped plots differ in nitrate content, on the average, about the same at each foot in depth.

Tables V and VI summarize the effect on soil nitrates of manure and moisture if considered separately.

TABLE V.—*Effect of manure on the nitrates in cropped and fallow soil. Average of all moisture treatments for three years*

[Results expressed as p. p. m. of sodium nitrate]

Depth of soil.	Total nitrates.							
	No manure.		5 tons of manure.		15 tons of manure.		Average.	
	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.
Feet.								
1	14.7	27.8	13.2	41.2	21.8	56.8	16.6	41.9
2	13.0	20.6	7.4	35.4	14.4	49.9	11.6	35.3
3	8.8	22.8	7.4	37.2	12.5	38.3	9.6	32.8
4	12.1	26.3	7.6	34.6	12.9	37 4	10.9	32.8
5	10.3	26.7	6.8	32.8	12.3	35.9	9.8	31.8
6	10.9	28.3	8.5	35.9	9.0	44.1	9.5	36.1
7	11.6	22.3	7.8	33.2	12.0	43.4	10.5	33.0
8	11.5	21.6	9.6	26.2	10.6	38.6	10.6	28.8
9	10.1	18.3	8.5	83.9	11.2	34.6	9.9	25.6
10	8.6	19.6	9.2	28.3	11.0	39.9	9.6	29.3
Average	11.2	23.4	8.6	32.9	12.8	41.9	10.9	32.7

TABLE VI.—*Effect of irrigation water on the nitrates in cropped and uncropped soil. Average of all manure treatments for three years*

[Results expressed as p. p. m. of sodium nitrate]

Depth (feet).	Water applied.													
	None.		5 inches.		10 inches.		20 inches.		30 inches.		40 inches.		Average.	
	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.
1	20.8	56.2	17.2	63.6	18.8	48.1	14.7	29.6	17.2	31.3	10.7	22.4	16.6	41.9
2	16.6	44.0	11.3	51.7	13.3	39.6	9.5	29.3	9.9	28.1	9.1	19.0	11.6	35.3
3	12.5	40.0	12.3	42.0	12.7	37.0	7.7	25.3	7.5	31.1	4.4	21.0	9.5	32.7
4	16.6	41.0	12.9	41.0	15.1	40.5	8.7	31.7	7.7	25.5	4.2	16.9	10.9	32.8
5	17.2	45.6	10.9	37.3	9.9	32.9	7.9	38.9	5.7	20.4	7.2	15.8	9.8	31.8
6	15.8	45.0	10.5	46.7	8.9	38.2	8.1	38.1	7.1	29.1	6.9	19.4	9.5	36.1
7	12.9	47.1	11.3	38.0	9.9	32.3	9.4	32.3	9.9	30.1	9.5	18.0	10.5	33.0
8	13.9	38.4	12.9	33.3	10.3	27.7	9.5	27.7	10.3	23.3	6.5	22.4	10.6	28.8
9	11.7	28.9	11.9	28.9	10.7	23.8	8.1	27.5	9.5	24.9	7.7	19.6	9.9	25.6
10	9.3	37.4	11.1	40.0	13.9	28.7	8.6	26.6	8.7	25.3	5.9	17.6	9.6	29.3
Average	14.7	42.5	12.2	42.3	12.3	34.7	9.2	30.8	9.4	26.9	7.2	19.2	10.8	32.7

RELATION OF PERCENTAGE OF WATER TO TOTAL SALTS AND NITRATES

To show the average effect of water applied, manure applied, and depth of soil on the moisture, total soluble salt, and nitrate content

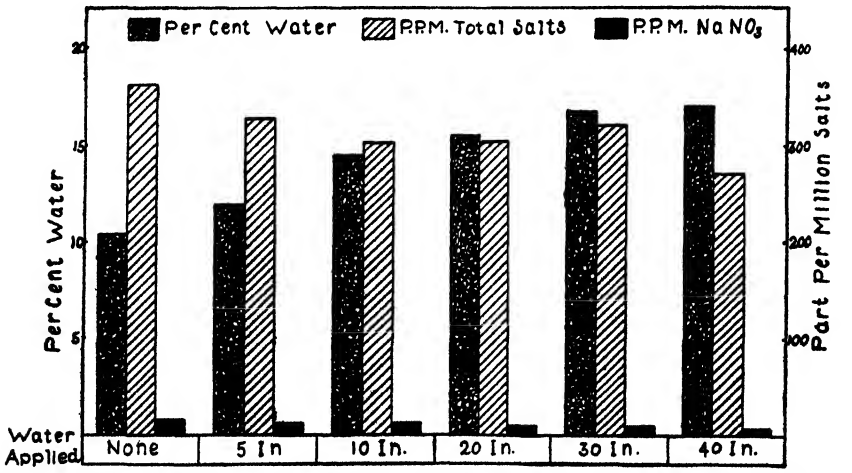

FIG. 13.—Graph showing effect of irrigation water on nitrates, total soluble salts, and percentage of moisture in cropped soil at the close of the cropping season. Average for all manuring treatments during three years to a depth of 10 feet.

of the soil, figures 13, 14, 15, 16, and 17 have been prepared. Some of the conclusions that might be drawn from these averages would not be substantiated when the factors influencing them were considered separately.

While the percentage moisture, total salts, and nitrates all decrease in the cropped soil receiving 5 tons of manure as compared with that receiving none, the total salts show a much greater proportional lessening

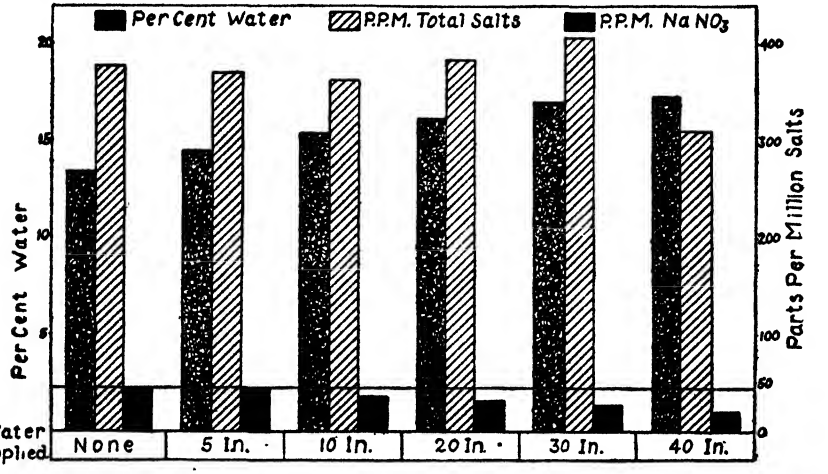

FIG. 14.—Graph showing effect of irrigation water on nitrates, total soluble salts, and percentage of moisture in fallow soil in the fall. Average for all manuring treatments during three years to a depth of 10 feet.

with no irrigation and an application of 5 inches of water than does that of the nitrates at the same treatments, while the moisture percentage decreases because of the manure, about the same amount for each

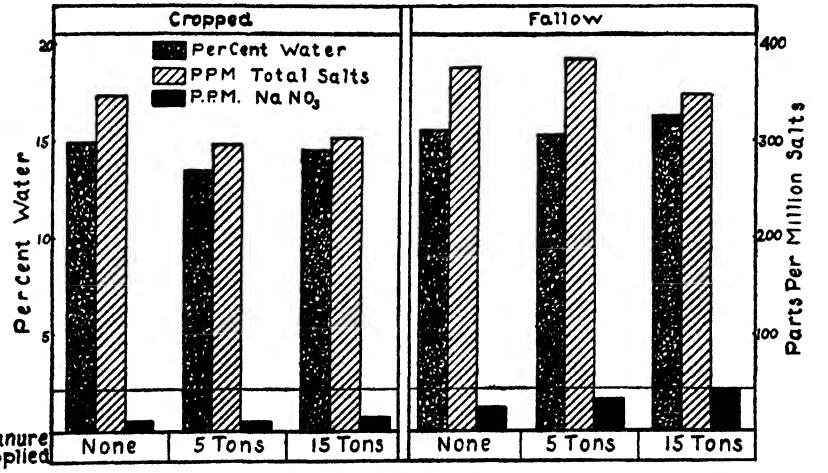

FIG. 15.—Graph showing effect of manure on nitrates, total soluble salts, and percentage of moisture cropped and fallow soil at the close of the cropping season. Average for all moisture treatments during three years to a depth of 10 feet.

irrigation treatment. These differences, while quite evenly distributed in depth for the total salts and nitrates, are greater at the fifth, sixth, and seventh feet for the percentage of moisture.

A comparison of the cropped soil receiving no manure with that to which 15 tons were applied shows the moisture to decrease with the application of this amount of manure for no water and 5 inches of water; the

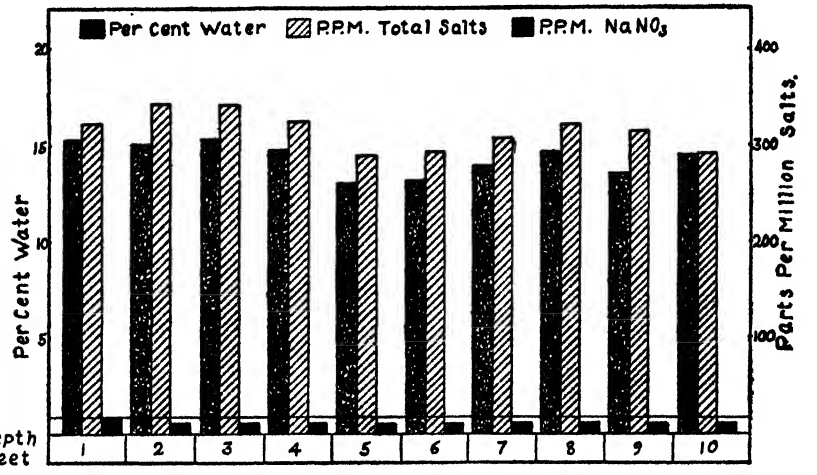

FIG. 16.—Graph showing nitrates, total soluble salts, and moisture at different depths in a cropped soil at the close of the cropping season. Average of all moisture and manuring treatments for three years.

total salts decrease with every irrigation treatment, but show the most marked effect where no water was applied; and the nitrates increase slightly and quite uniformly with all irrigation treatments. Downward,

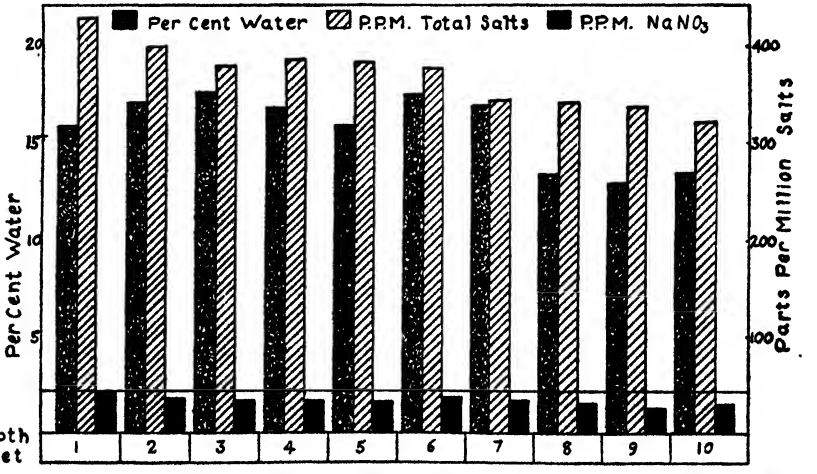

FIG. 17.—Graph showing nitrates, total soluble salts, and moisture at different depths in a fallow soil in the fall. Average of all moisture and manuring treatments for three years.

when averaged for all irrigations, the percentage of water was lower for the manuring only at the fourth, fifth, sixth, and seventh feet, where it was exceptionally low. The total salts of the lower feet of the manured

plots show the effects of the manure strongly, while with nitrates it is noticed most in the top feet.

On the average, moisture, total salts, and nitrates are greater for a manuring of 15 than for 5 tons. This effect is more noticeable for the percentage of moisture with the larger irrigation applications and for the total salts and nitrates with the smaller ones. The nitrates are affected proportionately more by the manure applied than are the total salts or moisture content. For each of the determinations the top feet are influenced more than the lower ones by the larger application, when compared with the 5-ton manuring.

When cropped plots are averaged for manure and depth the data show the moisture content to rise steadily with increasing quantities of irrigation water applied, while, in general, both total salts and nitrates decrease as the amount applied increases. In a comparison of the influence of the irrigations, the nitrates show the greatest effect of the water. The ratio of total salts to nitrates as sodium nitrate rises from 24.5 to 1 without water applied to 37.5 to 1 when 40 inches of irrigation water were used.

For the fallow plots receiving no manure and 5 tons the moisture content of the soil was higher where the manure was not added, while both the total salts and the nitrates were higher with the manure when the irrigation applications were 10 and 5 inches, or more, respectively. The manure affects the nitrates proportionately more than it does either the total salts or percentage water. The nitrates are influenced proportionately more in the top feet than the other two factors discussed here.

If the percentage of moisture in the soil where no water was applied be excepted, the moisture and nitrate content of the fallow plots receiving 15 tons of manure were higher than for those on which no manure was applied, the differences being proportionately greater for the nitrates. The nitrates for these treatments showed the influence of the manure most when the irrigation was 5 inches, and where no water was added. The effect is a little more noticeable in the top feet than lower. The total salts were greater in every case where the soil was not manured than where it received 15 tons to the acre.

The averages for nitrates and percentage of moisture are greater for the 15 than for the 5 tons of manure, although every irrigation treatment for nitrates does not show this tendency. Similar to the nitrates, the total salts increased more for the 15 tons of manure than with 5 tons only on the unirrigated and the 5-inch irrigated plots, but the average for the total salts favors the 5-ton application. The effect of manuring was especially noticeable for the total salts and nitrates in the upper feet, while with the percentage water the differences due to the manure were more evenly distributed for depth.

The averages for the fallow plots show an increase in the moisture content and a decline in the nitrates for increasing applications of water,

as was found for the cropped plots. The total salts do not show the tendency to decrease with increasing water, although the 40-inch applications caused a rather marked decrease over other moisture treatments.

The nitrate decline with increasing water was such that the ratio of the total salts to sodium nitrate increased from about 8.9 to 1 with no water applied to 16.2 to 1 where the application was 40 inches. The increase in the ratio of total salts to nitrates from 10.2 to 1 to 11.7 to 1 between the first and the fourth feet shows the nitrates to decrease more rapidly with increasing depth than do the total salts. It will be noticed that the ratio of total salts to nitric nitrogen is about 10 times what King found it to be for humid soils.

RELATION OF CROPS TO SALTS AND MOISTURE

Figure 18 gives the combined yields of grain and stover per acre for three years of this experiment.

TABLE VII.—*Summary of nitrates, total soluble salts, percentage of soil moisture, and yield of crops with different applications of manure. Average of all moisture treatments for three years*

Quantity of manure.	Moisture.		Sodium nitrate.		Total soluble salts.		Crop yields.
	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.	
	Per cent.	*Per cent.*	*P. p. m.*	*P. p. m.*	*P. p. m.*	*P. p. m.*	*Tons.*
None	14.9	15.5	11.2	23.4	345	377	4.106
5 tons	13.4	15.2	8.6	32.9	296	384	6.273
15 tons	14.5	16.2	12.8	41.9	302	347	7.053
Average	14.3	15.6	10.9	32.7	314	369	5.811

TABLE VIII.—*Summary of nitrates, total soluble salts, percentage of soil moisture, and yield of crops with different irrigation treatments. Average of all manurings for three years*

Water applied.	Moisture.		Sodium nitrate		Total soluble salts.		Crop yields.
	Cropped.	Fallow.	Cropped.	Fallow.	Cropped.	Fallow.	
	Per cent.	*Per cent.*	*P. p. m.*	*P. p. m.*	*P. m. p.*	*P. p. m.*	*Tons.*
None	10.3	13.4	14.7	42.5	360	378	5.155
5 inches	11.9	14.5	12.2	42.3	325	371	5.993
10 inches	14.4	15.4	12.3	34.7	307	363	6.026
20 inches	15.4	16.2	9.2	30.8	304	385	6.246
30 inches	16.7	17.0	9.4	26.9	320	407	5.891
40 inches	16.9	17.3	7.2	19.2	270	311	5.582
Average	14.3	15.6	10.8	32.7	314	369	5.811

In Tables VII and VIII are found the summarized results of the effect of manure and water, respectively, on the moisture, total salts, and nitrates found in the soils from which the crops were taken, compared with adjacent land on which no crops had been grown. The increase in crop is much greater in proportion for 5 tons than for 15. Except for the 5-inch application of water with 15 tons of manure, the largest yields were secured with 20 inches of water. The effect of cropping is felt

proportionately more for the nitrates than for the percentage moisture or total salts; the greatest effect of the crop on the moisture and total salts being felt on the plots which received 5 tons of manure, and for nitrates with 15 tons if we consider the difference in these factors between cropped and fallow plots to have all gone to the crop. The effect of the crop on the moisture content is more marked for no and 5 inches of water than for the larger amounts and is more for these quantities of water with 15 tons of manure than with the other manuring treatments.

The difference between cropped and fallow plots for nitrates is greater, as an average, with no and 5 inches of water, the loss due to the crop being greatest for 15 tons of manure.

In general, then, it may be said that, although cropping reduces the percentage of moisture, the total salts, and nitrates of the soil, the

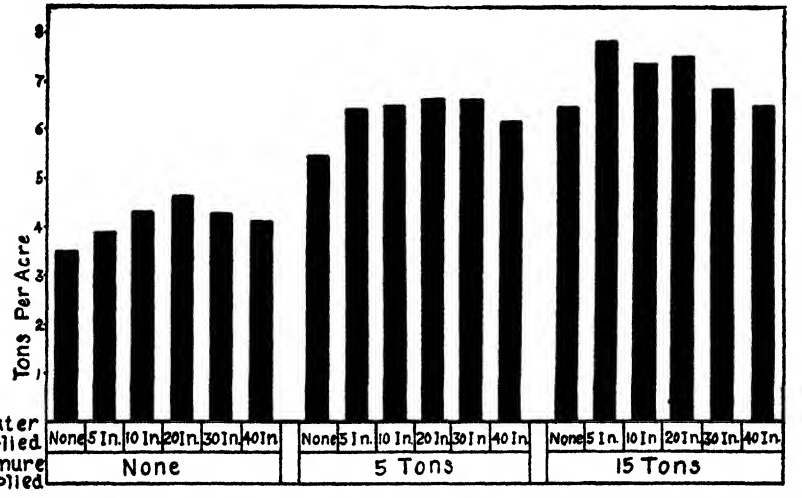

FIG. 18.—Graph showing the total yield of corn stover and grain produced with different irrigation and manuring treatments. Average for three years.

reduction does not seem to bear as close relationship to the crop yield as to the water and manure applied. More nitrates are formed with rather low percentages of water, and the plants seem to use more nitrate nitrogen where water is not plentiful than where more is given.

SUMMARY

Three distinct sets of experiments are reported in this paper.

With a sod soil held in the laboratory for 2½ years, the total salts and nitrates accumulated most rapidly with a moisture content between 23 and 28 per cent.

Cropped and uncropped soil kept in large tanks under controlled moisture conditions showed a decrease in nitrates and total soluble salts as the percentage of moisture increased, the nitrates being particularly low in water-logged soil.

Under field conditions more nitrates were found in both cropped and fallow soils during the summer than just after the corn crop was harvested.

The nitrates of the fallow field soils averaged higher with a manuring of either 5 or 15 tons to the acre than with no manure, but on the cropped soil, although the 15-ton application of manure resulted in more nitrates than no manure, the 5-ton did not. The fallow soil showed the effect of the manure on the nitrates more in the top 2 or 3 feet than at lower depths.

The highest soluble-salt content of fallow soil was on plots manured at the rate of 5 tons to the acre; the lowest was on plots receiving 15 tons. The corresponding high and low points in cropped soil were on plots receiving no manure and 5 tons to the acre, respectively.

Unirrigated land contained more nitrates than irrigated on both cropped and uncropped plots. Increasing the irrigation water applied to the soil decreased its nitrate content.

The total soluble salts in cropped plots decreased as the water applied increased and in fallow soil an application of 40 inches of water resulted in less salts than where no irrigation water was added.

The treatment affected the salts more in the surface foot than at greater depths with small irrigations, but when 20 inches of water or more were applied some of the salts seemed to have moved below 10 feet in depth.

Large irrigations decreased the soluble salts in cropped more rapidly than it did in fallow soils.

Manuring or irrigating the soil affected the nitrates relatively more than the total salts.

In unmanured soil the nitrate content was about twice as great with a fallow as with a crop, and in manured, it was about three times as great.

The ratio of total soluble salts to the quantity of sodium nitrate found in a cropped soil rose from 24.5 to 1 without irrigation to 37.5 to 1 when 40 inches of water were used. The ratio in fallow soil increased from 8.9 to 1 with no irrigation water to 16.2 to 1 with 40 inches.

The field results do not indicate a close relationship between the crop yield and the total soluble-salt or the nitrate content of the soil if the differences between cropped and fallow soils indicate the amounts of these substances which the crop used.

After a consideration of both pot and field experiments, it is evident that the soluble salts and especially the nitrates are found in lower concentrations in soils receiving large quantities of irrigation water than in those receiving less water. This is probably due in part to the leaching action of the heavy irrigations as well as to the lower nitrification in the presence of excessive soil moisture.

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THE RELATIVE INFLUENCE OF MICROORGANISMS AND PLANT ENZYMS ON THE FERMENTATION OF CORN SILAGE

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PRELIMINARY DISCUSSION

HISTORICAL REVIEW

Ever since the fermentation of silage has been studied and discussed the question of the agent causing the fermentation has been in controversy. Some investigators have made the statement, based on evidence more or less incomplete, that microorganisms are solely responsible for the changes undergone by the ensiled forage. Other workers, who have based their conclusions on equally incomplete data, have held that in silage produced under proper conditions bacteria and yeasts do not figure to any appreciable extent, but that the plant cell itself is the cause of the chemical changes which take place in its constituents. Still other writers have sometimes taken sides on the subject without presenting any new data bearing on the problem.

Among the earliest workers on the chemistry and biology of silage formation were Burrill and Manns (3),[1] who found many species of bacteria in the silage, and stated that they were the cause of the chemical changes. Babcock and Russell (1) made silage in the presence of chloroform, ether, and benzene, obtaining in each case a change of color, some increase in acidity, and typical silage odor and flavor. These results and deductions made from other observations on the gases of the silo, the number of bacteria found, and on silage made from mature and immature corn led them to believe that bacteria were nonessential and that the cause of the fermentation was mainly the intramolecular changes which occur in protoplasm under anaerobic conditions when ordinary metabolic processes are suspended. Harding (8), working under their direction, found bacteria but no constant flora in silage. E. J. Russell (17) came to the conclusion that the primary and essential changes in silage fermentation were brought about

[1] Reference is made by number to "Literature cited," p. 378–380

by the plant cell and its enzyms and that the changes caused by bacteria were secondary and nonessential. Esten and Mason (6) found such large numbers of bacteria and yeasts in silage that they considered them the only important factors concerned. Hunter and Bushnell (10) recently found large numbers of the *Bacterium bulgaricus* group in silage and considered their activities very important. All the work mentioned above has been done with silage made from the corn plant (*Zea mays*), which is the chief silage crop in this country.

Investigators in plant physiology have found evidence of the evolution of carbon dioxid and the formation of alcohol under aseptic conditions in the tissues of many plants, including maize. The distinction as to whether this is due to the action of enzyms within the cells or to respiratory activities of the cell protoplasm is not made; but similar respiratory changes appear to be common to the majority of plants, especially in their seeds. It has been suggested that the respiration of plants under anaerobic conditions is identical with alcoholic fermentation. In many cases some of the alcohol is further oxidized or otherwise changed, but the ratio of carbon dioxid to alcohol is often found to be comparable to that of ordinary alcoholic fermentation by the zymase of yeast. Of course, anaerobic conditions obtain in the silo after the first few hours. Doroféjew (4) found that the respiration of injured leaves was accelerated. This may have some significance in connection with the chopping of corn before it is ensiled. Zaleski and Reinhard (27) and others have noticed similar effects in wounded vegetable tissues. With various seeds placed under anaerobic conditions Godlewski and Polzeniusz (7), Stoklasa et al. (20, 21), Minenkoff (13), and a number of others have found that alcohol and carbon dioxid are produced. Similar results have been obtained by others with other plant tissues than the seeds. Although Mazé and Perrier (12) have questioned the results of Stoklasa, it is still possible that such respiratory activities play a part in the formation of silage.

Certain enzyms have been shown to be present in corn grain. A proteoclastic enzym has been found by Vines (24) and likewise by Scheunert and Grimmer (18), who found an amylase also present. Sigmund (23) found a lipase in both the resting and germinating seeds of maize. Price (15) demonstrated the presence of a peroxidase, a catalase, a protease, an invertase, and a glucosidase in cornstalks. White (26) found proteoclastic and amyloclastic enzyms in maize seeds, which retained their activity in seeds 20 years old.

STATEMENT OF PROBLEM

A sharp differentiation between the activities of enzyms and microorganisms in a given medium is practically impossible. Both are susceptible to injury and destruction by heat and are more or less similarly subject to the inhibitive effect of our common antiseptics. Moreover,

some of the principal manifestations of bacterial activity are identical with those of enzym action, as, for example, the evolution of carbon dioxid, production of alcohol, rise in temperature of medium, and hydrolysis of protein. Antiseptics, if used in high enough concentration to inhibit all bacterial growth, seem also to exercise a deleterious effect on the plant tissues and their enzyms. Aseptic conditions can be maintained for respiration experiments on small amounts of plant tissue, but it would be very difficult to produce silage under such conditions. The problem attacked was therefore to differentiate as accurately as possible between the results of the various activities of these two kinds of agents. A number of different experimental methods were employed in the effort to arrive at a distinction between them.

EXPERIMENTAL METHODS

Silage made in the laboratory in glass jars has been used in the greater part of this work. Experimental conditions can in this way be easily controlled, and comparisons are thus possible. The corn was chopped in a small silage cutter or was taken from the college farm silage cutter and packed as tightly as possible into cylindrical wide-mouth jars which were closed with rubber stoppers. Each was provided with an outlet tube for excess gases, which was closed with a pinchcock. Silage made in this manner is perfectly and normally preserved with characteristic appearance and aroma. Comparisons of chemical data between silage from the farm silos and from laboratory silos show no considerable difference. Of course, no two lots of silage are ever exactly alike chemically. The writer has previously made both corn silage and mixed silages in this manner (9, 11). Results obtained show no evidence that this laboratory silage is essentially different from silage made from similar material in an air-tight farm silo.

Some of the corn used in this work was grown to maturity in the greenhouse. Both greenhouse corn and field-grown corn were used, to prevent any possible abnormal results. The greenhouse corn was generally nearly as good in quality as the field-grown corn.

The analytical methods used are based upon the characteristic chemical changes which take place in silage fermentation. The formation of acids and alcohols, the evolution of carbon dioxid, the disappearance of simple sugars, and the degradation of protein, which are the principal chemical phenomena of the fermentation, have been measured by the methods described below. Results from methods based on these chemical changes show, as the conventional estimations of crude protein, fiber, ether extract, etc. do not, the nature and extent of fermentation and the character of the silage, as nearly as chemical analysis can show. In each case comparisons were made with similar figures obtained on samples of the green corn from which the silage was made. In each case the chemical determinations were made upon the juice expressed

from the silage in a Buchner press, under a pressure of 300 to 400 kgm. per square centimeter. This method of sampling facilitates the chemical examination, insures a well-mixed sample, and makes possible more comparable results.

The methods used are as follows:

TOTAL ACIDITY.—Ten c. c. of silage juice were diluted to about 500 c. c. with carbon dioxid free water, and titrated with decinormal barium hydroxid solution in the presence of phenolphthalein till a distinct pink appeared by reflected light against a white background.

VOLATILE ACIDITY.—One hundred c. c. of juice were distilled in a current of carbon dioxid free steam. To hasten the liberation of volatile acids and alcohols, 100 gm. of sodium chlorid were added to the juice. About 600 c. c. of distillate were titrated with baryta water in the presence of phenolphthalein.

ALCOHOLS (Distillation method).—The distillate from the volatile acid determination was neutralized with baryta water (solid phenolphthalein being added) and concentrated by repeated distillation with sodium chlorid (2). About 50 c. c. of alcohol solution were oxidized [1] in a pressure flask in a boiling water bath for 30 to 40 minutes, and the volatile acids then distilled off four or five times, with additions of carbon dioxid free water. The volatile acids were titrated and calculated as ethyl alcohol.

ALCOHOLS (Aeration method).—In this method (21) a current of air was drawn through the silage juice, which was saturated with ammonium sulphate, into concentrated sulphuric acid. The sulphuric acid-alcohol solution was then oxidized with the potassium dichromate solution, and distilled and calculated as in the previous method. Although other alcohols are formed in silage in small amounts, all were calculated as ethyl alcohol.

TOTAL SUGARS.—Fifty c. c. of juice were clarified with neutral lead acetate, the excess lead precipitated with anhydrous sodium carbonate, an aliquot allowed to stand 24 hours with hydrochloric acid, neutralized, and the total reducing sugars determined on an aliquot by either the Defren-O'Sullivan method or the slightly modified volumetric method of Schoorl (19). In any given series the same method was used to insure comparable results.

AMINO NITROGEN.—The amino nitrogen was determined on the diluted juice with the Van Slyke apparatus (22). This determination shows the relative degree of hydrolysis of protein if used on the same or similar material at successive periods.

AMMONIA NITROGEN.—A 50-c.c. sample of the juice was distilled with magnesium oxid, according to the official fertilizer method.

MOISTURE.—Samples of about 100 gm. of silage were dried to constant weight, in most cases in a vacuum oven at 60° C.

[1] The oxidizing solution used was made up in the following proportions: 10 gm. of potassium dichromate ($K_2Cr_2O_7$), 20 gm. of sulphuric acid (H_2SO_4), 70 gm. of water.

SILAGE MADE UNDER ANTISEPTIC CONDITIONS

Corn silage made in the presence of ether and chloroform, as done by Babcock and Russell (1), is, of course, well preserved and evolves, after the antiseptic has been allowed to evaporate, an odor quite aromatic and characteristic of silage. The exact amount of antiseptic which should be added to inhibit bacterial growth without seriously impairing enzymic action is, however, very difficult to estimate. Moreover, Wagner (25) has found that certain bacteria may flourish in the presence of benzene, phloroglucinol, phenol, and phenolic derivatives. Some experimental silage was made, however, in the presence of chloroform, toluene, and cresol. Analytical data on these are shown in Table I.

TABLE I.—*Analytical data on antiseptic silage*

[Calculated on basis of 100 gm. of dry silage.]

Kind of silage.	Total acidity (*N*/10).	Alcohol.	NH_2—N.
	C. c.	*Gm.*	*Gm.*
Typical green corn	34	0	0.052
Toluene silage (2 per cent)	31	Trace.	.057
Chloroform silage (5 per cent)	250	.....	.176
Cresol silage (0.5 per cent)	255	0.15	.199
Normal silage	675	.81	.256

The amount of toluene added seems to have been enough to stop practically all change. No bacterial growth was obtained from the chloroform silage. It seems likely that the jar of cresol silage contained a limited number of active organisms, as two forms were isolated from this silage, one of them an acid former, and no evidence of spore formation could be obtained with either of these organisms. Some of the results of the work with antiseptics have been introduced here to show the apparent impossibility of obtaining conclusive results, at least with silage, by using such methods alone. These data, however, may be of some value when compared to other data shown on the following pages. Further experiments with varying amounts of antiseptics were not attempted, as it seemed likely that other methods would give more conclusive results.

OTHER EXPERIMENTAL SILAGE

An effort was made to learn the effect of bacteria and yeasts alone, by heating jars of chopped corn to destroy the enzyms, followed by inoculation with an infusion of normal silage, which should carry the normal mixed flora of silage. After inoculation the jars were in each case incubated at 28° to 30° C., with a control jar of normal silage made from the same sample of corn. The analytical data, which are interesting but not conclusive, are shown in Table II.

TABLE II.—*Silage heated and inoculated*

[Data on 100 c. c. of juice]

Kind of silage.	Total acidity (*N*/10).	Volatile acidity (*N*/10).	Alcohol.	NH_3-N
	C. c.	*C. c.*	*Gm.*	*Gm.*
Green corn	23	1.8	0	0.044
Normal silage (control)	375	118.0	.265	.....
Corn heated to 80° and inoculated	274	123.6	.343	.135
Green corn	43	.....	0	.111
Normal silage	318	109.6	.150	.214
Corn heated to 90° and inoculated	198	99.1	.244	.068
Green corn	19.5	1.2	0	.024
Normal silage	342.5	73.1	.297	.114
Corn heated to 85° and inoculated	301.5	58.4	.188	.035

The prominent part which may be played by yeasts in the fermentation of silage under certain conditions was demonstrated by adding to a jar of silage sufficient tartaric acid in solution to make 2 per cent of the weight of the silage. An acid mixture of this strength practically inhibits bacterial action and favors the development of yeasts. A comparison of the acidity of the silage with the amount of tartaric acid added showed that evidently no other acid had been formed, except some volatile acid, which might possibly have come from the oxidation of alcohol. The quantity of alcohol found in 100 c. c. of this silage juice was 1.746 gm., expressed as ethyl alcohol, while normal silage juice contains only from 0.20 to 0.45 gm.

RATE OF CHEMICAL CHANGES IN SILAGE FERMENTATION

A more conclusive method of differentiating between the activities of enzyms and of microorganisms was suggested by a paper by Rahn (16), who discussed the usefulness of curves in the interpretation of microbial and biochemical processes. It is shown by Rahn that the curve which is obtained when the formation of products of fermentation or other biochemical process is plotted, taking as abscissæ the total time elapsed and as ordinates the total amounts of compounds produced, is in many cases indicative of the nature or cause of the change. If the change is caused by enzymic action and is, therefore, purely chemical, the active mass of the agent causing the change does not increase as the reaction progresses; and the decreasing concentration of the substance acted upon and the accumulation of end products tend to decrease the rate of change. Thus, the curve becomes convex toward the Y axis. The mass of enzym does not increase unless there are living cells present to elaborate more enzym. However, if organisms are present and are active, they multiply until the exhaustion of nutrients or the accumulation of end products retards and finally stops their increase. Until this time the

rate of change increases with the increase in number of organisms, and the resulting curve is convex toward the X axis. Then there is a point of inflection, after which the curve becomes similar to the enzymic curve. An example of a curve of this type is shown in figure 1, a typical "fermentation curve." There is always a point of inflection, or change in direction of curvature in a bacterial curve, provided the data begin before the number of organisms has reached its maximum. These two kinds of curves are discussed at some length by Rahn in the article cited (16).

In natural or mixed fermentations, such as the formation of silage, it is possible that different processes taking place at the same time will destroy the natural form of the curve. Or if both chemical and biological factors are present and producing the same substance, e. g., alcohol, the nature of the curve might be variable, depending upon the relative "active mass" of the two agents. In the case of so large an inoculation that the bacteria do not multiply materially, a bacterial curve might possibly resemble the curve of an enzymatic process. However, the chemical composition of the material and the extent of the fermentation depend upon many variable factors, such as soil and meteorological conditions, the method and rate of filling the silo, and opportunity for inoculation. The silage resulting is thus a variable product, depending on these and other factors. Therefore it seems very unlikely that any considerable error in the interpretation of curves drawn from silage data would persist through a number of entirely separate experiments. This is evident from the data given in the following pages.

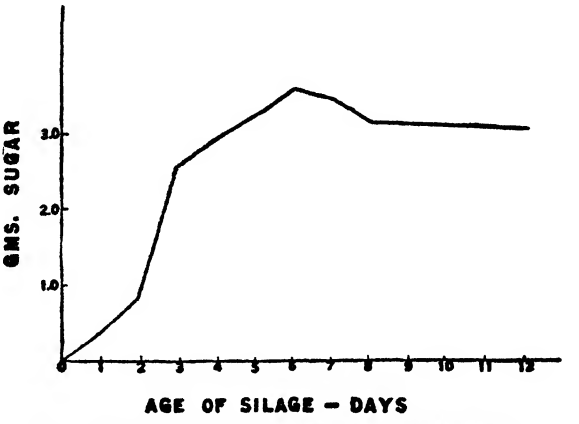

FIG 1.—Curve showing the disappearance of sugars in series 1.

The analytical methods outlined above were used to obtain data on the chemical changes occurring during the early period of silage fermentation, in order that curves such as just described might be plotted. The data obtained by Neidig (14) on this part of the fermentation process indicate the impossibility of obtaining regular curves and strictly comparable results by taking samples from a farm silo, owing to the variability in composition of the silage in different parts of the silo, and the necessity of the perfect exclusion of air throughout the process. Therefore, silage was made in the laboratory in small jars as mentioned above. The chopped corn was very thoroughly mixed, and after a sample had been taken for the initial analysis, it was packed tightly into jars.

The jars were kept under the same conditions, generally in an incubator at 28° to 32° C., following the average rise of temperature in the silo. One jar was opened each day and the juice pressed out and analyzed. This method should give as nearly correct results on the rate of change as it is possible to obtain, it being granted that silage formation in small jars is perfectly normal. The changes which were found were very similar to the changes observed by Neidig in the farm silos, except that the uniformity of the samples gave much more uniform and regular curves.

TABLE III.—*Series 1: Formation of acid and alcohol, disappearance of sugars, and increase in animo nitrogen in silage*

[All data calculated to sample of 100 c. c. of silage juice.]

Age of silage.	Total acidity *N/10* sol.	Alcohol.	NH_3—N.	Total sugars.	Total sugars which disappeared.
Days.	*C. c.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
0	27.5	0.002	0.069	5.299	0.000
1	47.5	.144	.083	4.934	.365
2	99.7	.065	.089	4.526	.773
3	166.2	.067	.098	2.774	2.525
4	190.0	.082	.089	2.323	2.976
5	233.7	.090	.106	2.078	3.221
6	253.6	.129	.108	1.742	3.557
7	278.3	.095	.109	1.867	3.432
8	226.1	.082	.109	2.203	3.096
12	228.0	.114	.109	2.270	3.029

A series of nine jars of silage (series 1), using corn grown to maturity in the greenhouse, gave very interesting results. The corn was mixed and ensiled as described above. The determinations on the green corn and on each day's sample of silage juice were made, as usual, in a strictly comparable manner. The analytical data are given in Table III.

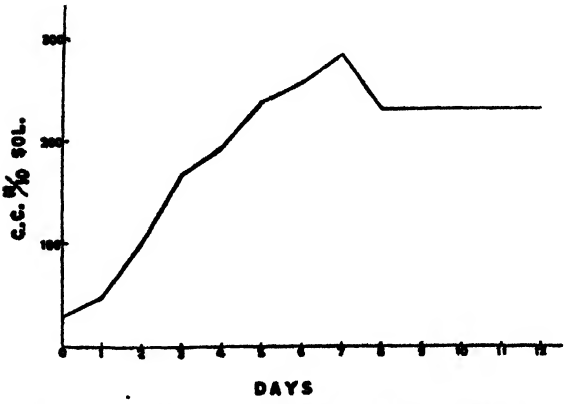

FIG. 2.—Curve showing the development of acidity in series 1.

The curves plotted from the data in Table III are shown in figures 1 to 4. The curves showing the disappearance of sugars and formation of acids are similar in shape and are typical of bacterial fermentation. The amino-nitrogen curve, which shows the rate of hydrolysis of protein, and the alcohol curve both show the abrupt rise at the beginning which characterizes the enzymic type of

curve. However, between the second and third days during the same period in which there appears a marked increase in bacterial activity in the sugar and acid curves, there is also a second rise in the NH_2–N curve, which is probable evidence of some proteoclastic action by bacteria.

A similar series of determinations (series 2) made two months later shows a somewhat different set of curves. Field-grown corn at the proper stage of maturity was chopped in the silage cutter, inoculated with material carrying the usual flora of the farm silage cutter, and ensiled as before. In this series total and volatile acid, alcohol, sugars, amino nitrogen, and ammonia nitrogen were determined. The analytical data are shown in Table IV.

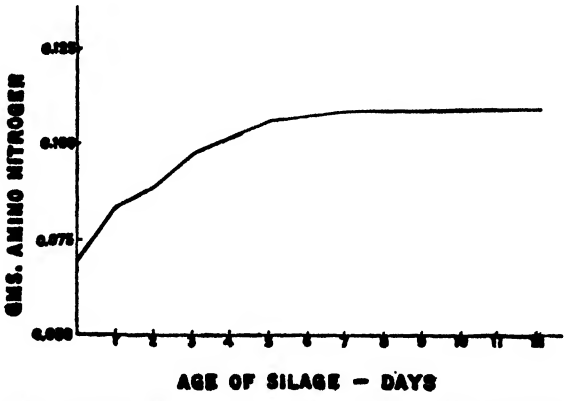

FIG. 3.—Curve showing the increase in amino nitrogen (and the rate of hydrolysis of protein) in series 1

TABLE IV.—*Series 2: Formation of total and volatile acid and alcohol, and the disappearance of sugars, amino nitrogen, and ammonia nitrogen in silage*

[Data on 100 c. c. of silage juice.]

Age of silage	Total acidity (*N*/10).	Volatile acidity (*N*/10).	Alcohol.	NH_2-N.	NH_3-N.	Total sugars.	Disappearance of sugars.
Days.	*C. c.*	*C. c.*	*Gm.*	*Gm*	*Gm*	*Gm.*	*Gm.*
0	17. 3	1. 4	0. 000	0. 021	0. 006	3. 139	0. 000
1	97. 0	42. 1	. 188	. 053	. 008	1. 622	1. 517
2	160. 3	56. 9	. 210	. 060	. 010	1. 008	2. 131
3	205. 4	65. 2	. 197	. 066	. 010	. 648	2. 491
4	235. 2	68. 4	. 262	. 095	. 026	. 235	2. 904
5	250. 6	68. 6	. 307	. 098	. 018	. 158	2. 981
7	254. 4	70. 6	. 238	. 097	. 019	. 019	3. 120
9	263. 0	71. 0	. 403	. 104	. 021	. 019	.....
12	266. 9	78. 9	. 256	. 084	. 019	. 120	.....
15	274. 6	75. 8	. 347	. 112	. 025	. 161	.....
21	291. 8	84. 0	. 354	. 128	. 027	....	.....
30	296. 6	79. 5	. 337	. 149	. 028	.....	.....

The form of the acid curves of this series (fig. 5), when compared to the usual form of the acid curves, suggests the possibility of so large an inoculation with acid-forming bacteria that the maximum in numbers was reached during the first 24 hours. The curve showing the disappearance of sugars (fig. 6) has the same form. The curve showing the formation of alcohol (fig. 4) is of the enzymic form, like the corresponding curve in series 1. Irregularities in these two alcohol curves

suggest the possibility that each is the resultant of the formation of alcohol by one or more agents and its simultaneous oxidation by other agents. Or there might possibly be some variation between individual samples in the series. The amino-nitrogen curve (fig. 7) shows an evident enzymatic protein hydrolysis during the first three days. Between the third and fourth days, however, an abrupt rise takes place, which bears out the assumption made from series 1, viz, that bacteria figure in the hydrolysis of protein after the first two or three days. It is noteworthy that a similar rise takes place during the same period in the sugar, alcohol, and ammonia-nitrogen curves, indicating a general increase in the activity of microorganisms at that time. As previously mentioned, just such a simultaneous rise was noticed in three of the curves in series 1 between the second and third days. This observation lends strength to the evidence in favor of enzymic action in these cases during the first two days.

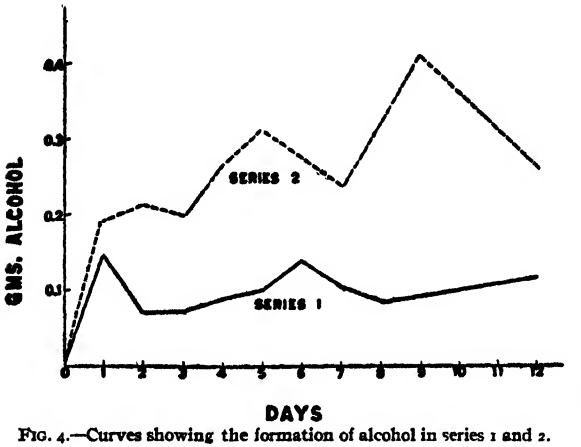

FIG. 4.—Curves showing the formation of alcohol in series 1 and 2.

Corn grown in the greenhouse was used for series 3. This corn made an excellent growth and was practically as good in quality as field-grown corn. The corn was chopped in the laboratory and ensiled as before, but with little opportunity for inoculation. Analytical data on this series are shown in Table V. The first jar of silage was opened when only 12 hours old.

TABLE V.—*Series 3: Formation of acids and alcohol and disappearance of sugars*

[Data on 100 c. c of silage juice]

Age of silage.	Total acidity (*N*/10).	Volatile acidity (*N*/10).	Alcohol.	Total sugars.	Disappearance of sugars.
Days	*C. c.*	*C. c.*	*Gm.*	*Gm.*	*Gm.*
0	14.0	2.7	0.001	3.850	0
½	25.0	2.8	.079	4.292	− .442
1	36.5	2.9	.123	4.060	− .210
2	39.0	3.0	.272	3.428	+ .422
4	41.5	3.1	.312	2.236	1.614
7	63.5	5.0	.414	1.660	2.190

The increase in the amount of sugars during the first day is interesting, and is very probably due to the presence in the corn grain of amylase,

which in this case produced sugar from higher carbohydrates faster than the sugar was used. The development of acids is remarkably small, due perhaps to the slight opportunity for inoculation with acid-forming organisms. It is unfortunate that a larger number of jars was not filled in this experiment so that the later progress of acid formation could have been followed. Since there is no possibility in this series that the formation of acetic acid complicates the question of alcohol formation, some interesting observations are possible. The alcohol curve (fig. 8) after beginning like an enzymic curve takes another abrupt rise between the first and second days, probably when the yeasts become more active. The concomitant production of carbon dioxid in the silage of this series was also measured. (The data are given on page 374.) The carbon-dioxid curve (fig. 13) shows the same trend throughout as the alcohol curve, including the rise just mentioned between the first and second days. The carbon dioxid : alcohol ratios show a general increase as the fermentation progresses, as follows:

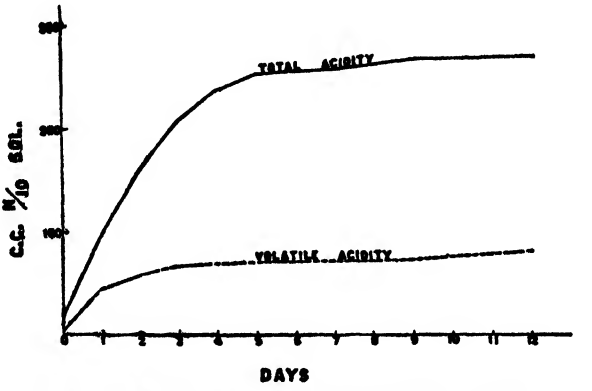

FIG. 5 —Curves showing the development of acidity in series 2

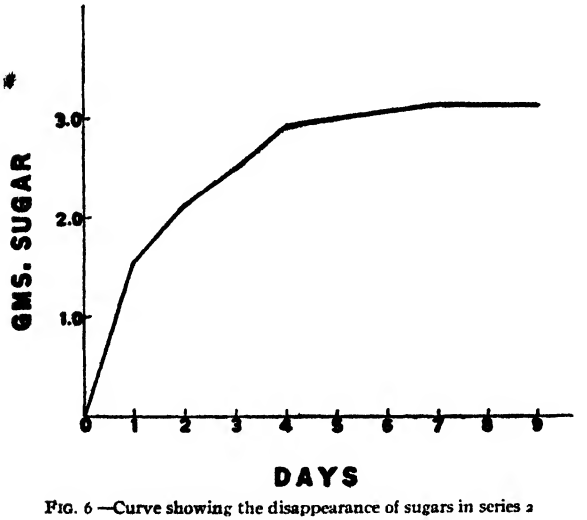

FIG. 6 —Curve showing the disappearance of sugars in series 2

Age of silage (days)	½	1	2	4	7
Ratio	1:0.51	1:0.61	1:0.86	1:0.79	1:0.97

This increase might be either because the yeasts are taking an increasingly greater part in the fermentation and the ratio therefore approaches the ratio of the ordinary alcoholic fermentation, or because carbon dioxid from other reactions is included in the amount evolved during the first few days. Of course, the above is the result of but a single experiment on this point.

In the next series (series 4) the corn used was from the same greenhouse plot as the preceding. The use of the same tools, etc., a week later, gave opportunity for a much larger inoculation with acid-forming bacteria. This is evidenced by the data shown below. In this series bacteriological counts of some of the samples were made.[1] The technic used was as follows: The sample was ground in a sterile mortar for 15 minutes, 50 gm. weighed out, placed in a liter flask with 500 c. c. of sterile water and shaken 100 times. One c. c. was plated in various dilutions in a yeast-extract agar and incubated for 48 hours at 37° C. Data on this series are given in Table VI.

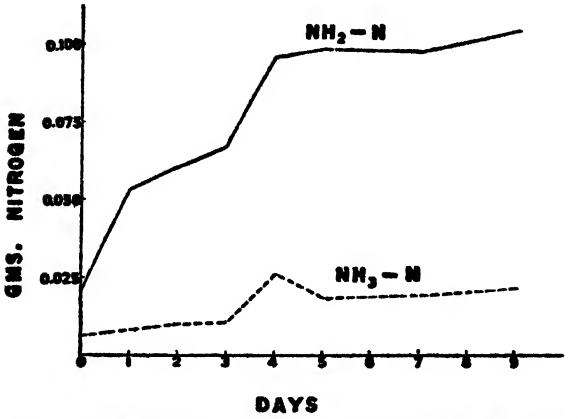

FIG. 7.—Curves showing the increase in amino nitrogen and ammonia nitrogen in series 2.

TABLE VI.—*Series 4: Formation of acids and alcohol, disappearance of sugars, amino nitrogen, and bacterial counts in silage*

[Data all on 100 c. c. of juice, except bacterial counts.]

Age of silage.	Total acidity (*N*/10).	Volatile acidity (*N*/10).	Alcohol.	NH_2—N	Total sugars.	Disappearance of sugars.	Bacteria per gram of silage.
Days.	*C. c.*	*C. c.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	
0	15. 5	2. 7	0. 001	0. 028	6. 662	0	.....
½	39. 0	3. 5	. 109	. 058	7. 036	— . 374	9, 900, 000
1	55. 5	4. 6	. 176	. 076	7. 594	— . 932	.....
2	121. 0	7. 1	. 192	. 108	5. 030	(+)1. 632	106, 500, 000
3	156. 0	18. 9	. 193	. 120	3. 764	2. 898	119, 000, 000
4	148. 5	24. 5	. 182	. 116	4. 636	2. 026	38, 800, 000
6	223. 5	47. 8	. 197	. 101	2. 324	4. 338	.....
9	254. 0	58. 2	. 268	. 107	2. 190	4. 472	106, 000, 000

It should be noted that the preliminary increase in sugars occurs as it did in series 3. The comparatively small bacterial count in the 4-day-old silage is reflected in the total acid, amino nitrogen, and sugar columns. The alcohol curve (fig. 8) shows the same enzymic form as before. The acid curves (fig. 9) show the usual bacterial form. The amino-nitrogen curve (fig. 10) is of the enzymic form as before. The curves showing the disappearance of sugar in series 3 and 4, though not reproduced here, approach the bacterial form.

[1] Acknowledgment is gratefully made to Dr. R. E. Buchanan for the use of laboratory facilities and media for making these counts.

A final lot of silage (series 5) was made from corn grown in the field during the present year. This corn lay in the field or on wagons 15 to 20 hours after cutting. It was taken from the farm silage cutter, mixed, and ensiled as before. The data are given in Table VII.

TABLE VII.—*Series 5: Formation of acids and alcohol in silage*

[Data on 100 c. c. of juice]

Age of silage.	Total acidity (*N*/10).	Volatile acidity (*N*/10).	Alcohol.
Days.	*C. c.*	*C. c.*	*Gm.*
0	45.0	6.0	0.022
½	47.5	6.5	.190
1	123.5	7.5	.269
2	218.5	36.8	.342
3	263.0	45.3	.515
4	312.5	65.2	.465
7	362.5	96.8	.633

The initial acid and alcohol content are rather high, as the corn stood so long after cutting. The acid curves (fig. 11) show the usual bacterial form and the alcohol curve (fig. 12) the usual enzymic form, with the later rise presumably due to yeasts.

The data obtained from these five series of experimental silage will be discussed below.

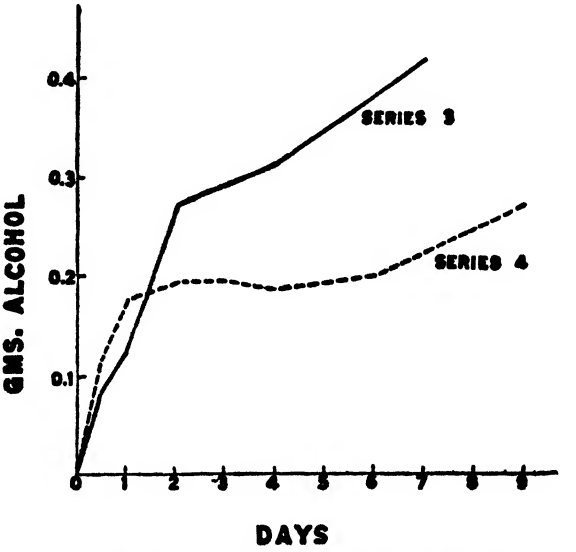

FIG. 8.—Curves showing the formation of alcohol in series 3 and 4.

EVOLUTION OF CARBON DIOXID

The amount of carbon dioxid evolved by silage, a constant and characteristic phenomenon of the process, was measured by absorption in caustic potash solution. The silage was packed into cylindrical specimen jars with wide mouths which were fitted with specially made rubber stoppers. A ⅛-inch galvanized-iron pipe was led to the bottom of each jar and the corn was tightly packed around it. This pipe was closed at the top with a rubber tube and a pinchcock. An outlet tube at the top of each jar was connected to an absorption train. The gas was forced through the train by its own pressure, which was always greatest during

the first day, gradually decreasing thereafter. At the end of each period the iron inlet tube of one of the jars was connected to a soda-lime tube and a current of air was drawn through the absorption train for 30 minutes to remove the carbon dioxid remaining in the jar. The silage could then be removed for analysis. The data from the three experiments are given in Table VIII.

TABLE VIII.—*Evolution of carbon dioxid in silage*

[All data calculated to sample of 100 gm. of silage.]

Age of silage.	Evolution of carbon dioxid.[a]		
	Experiment 1 ($Gm. CO_2$).	Experiment 2 ($Gm. CO_2$).	Experiment 3 ($Gm. CO_2$).
Days.			
½	.....	.....	0.115
1	0.288	0.228	.148
2	(b)	.404	.226
3	.341	.490	.....
4	(b)	(b)	.292
5	(b)	(b)	.....
6	.....	.508	.....
7	.....	.....	.315

[a] These data are from series 3, p. 370. [b] Determination lost.

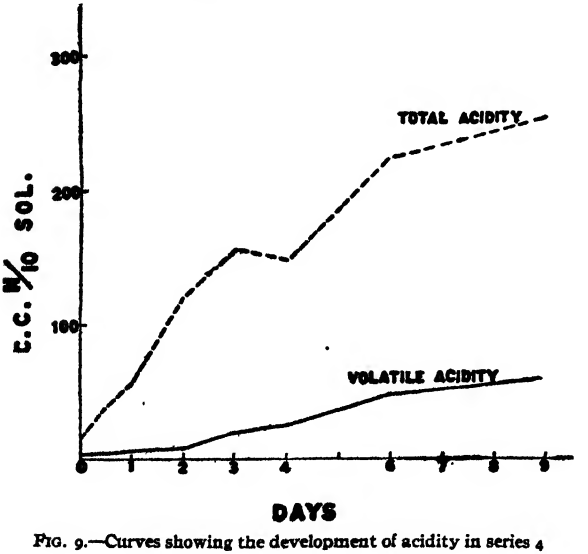

FIG. 9.—Curves showing the development of acidity in series 4

The curves plotted from these data are shown in figure 13. All are of the enzymic form, and check with the observations of the writer on all the silage he has made, viz, that the evolution of gas is always greatest during the first day or two, and nearly ceases after about four days. In most cases the rate of evolution is evidently kept up after the first day or two by contributions from bacteria and yeasts. In experiment 3 the curve shows a change of direction and a distinct rise during the second day, coincident, as remarked above, with a similar rise in the alcohol curve in the same experiment (cf. series 3).

RISE IN TEMPERATURE

Another characteristic phenomenon of silage fermentation, but one very much misunderstood in the early days of silage making, is the rise in temperature of the silage. Temperatures as high as 130° F. have been observed in the silo at or near the surface of the silage. This excessive heating is due to activity of microorganisms greatly accelerated by the presence of atmospheric oxygen, and occurs whenever silage is uncovered and left exposed to the air for a time. The temperature deep in the silo, however, protected from the air and sufficiently removed from the conduction of heat from the surface of the silage, is rarely higher than 80° to 90° F. It is rather unsatisfactory to attempt to obtain curves characteristic of bacterial or enzymic fermentation from the rise in temperature of the medium, on account of the number of somewhat extraneous factors involved. The outside temperature is always a factor, and the rise in temperature of the silage might easily affect the rate of chemical reactions or of bacterial growth, thus increasing the rate of temperature rise and perhaps changing the nature of the curve. Data which have been obtained from the farm silos[1] and from very carefully insulated laboratory silos suggest that the greater part of the heat developed is due to microbial action. It is considered unnecessary to reproduce these data here, as similar data have been published (14). However, one table is subjoined (Table IX) showing the rise in temperature at the surface of the silage in one of the farm silos. An iron pipe was forced down into the silage for about 4 feet, and a thermometer, immersed in a test tube full of water, lowered into the pipe so that the bulb was 2 feet below the surface of the silage. The top of the pipe was closed except when the thermometer was pulled up for reading. These data when plotted give a smooth and typical bacterial fermentation curve. Of course, this does not exclude the possibility of some heat production by enzym action.

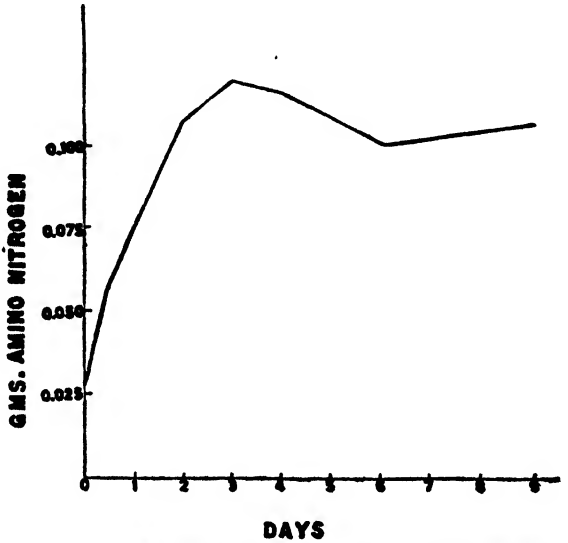

FIG. 10.—Curve showing the increase in amino nitrogen in series 4

[1] With the cooperation of the Agricultural Engineering Section of the Iowa Station.

TABLE IX.—*Rate of heating at surface of silage in brick silo*

Date.	Time.	Age of silage.	Temperature.	Outside temperature.
		Days.	°F.	°F.
Sept. 19	4 p. m.	0	71.2	61
Do	12 mid.		75.4	
Sept. 20	9 a. m.		82.0	67
Do	12 m.		85.6	
Do	6 p. m.	1	100.8	
Sept. 21	9 a. m.		113.4	58
Do	12 m.		115.3	
Do	4 p. m.	2	117.6	
Sept. 22	9 a. m.		122.2	53
Do	6 p. m.	3	123.2	
Sept. 23	12 m.	4	125.6	52
Sept. 24	12 m.	5	127.2	61
Sept. 25	12 m.	6	128.8	62

DISCUSSION AND CONCLUSIONS

It appears that neither microorganisms nor plant enzyms are alone responsible for the changes which take place in corn silage fermentation. The curves in these pages show that acid production is mainly if not entirely a phenomenon of bacterial activity in the silage. The results from the other experimental silage described above also suggest that the greater part of the acid is produced by microorganisms.

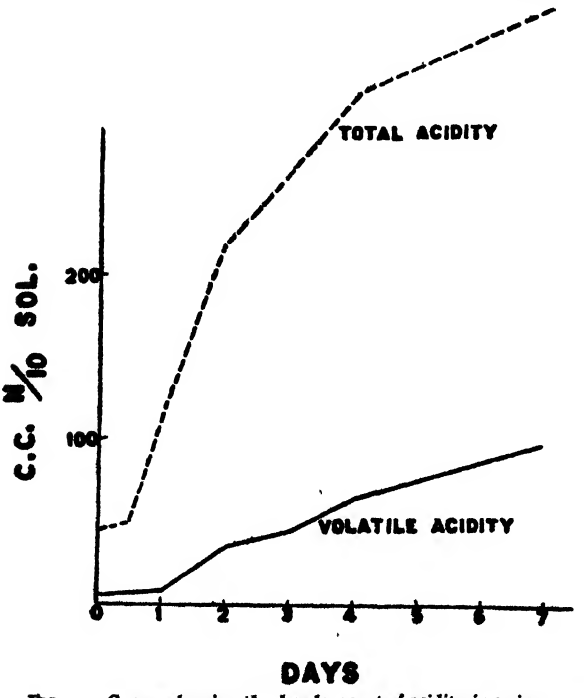

FIG. 11.—Curves showing the development of acidity in series 5

The curves showing the disappearance of sugars are, like the acid curves, generally of the bacterial fermentation type. Although some of the sugar is undoubtedly changed by enzym action, the greater amount seems to be metabolized by bacteria and yeasts.

The formation of alcohol, however, is evidently a phenomenon primarily of the respiratory or enzymatic activity of the plant cells. This is

suggested by investigators in plant physiology, who have often found zymase in plants, and is corroborated by the nature of the alcohol curves shown. The curves suggest a later production of alcohol by yeasts, and results from the other experimental silage support this idea. As stated above, Esten and Mason (6) found large numbers of yeasts in corn silage. Both factors, therefore, probably have a share in the alcohol production.

A similar statement holds good for the hydrolysis of protein as indicated by the amino-nitrogen content of the silage. Proteoclastic enzyms are present in corn grain, and the curves show evidence of their activity. Both the later rise in the amino-nitrogen curves noted above and the results from silage in which the enzyms were destroyed show some proteoclastic activity by microorganisms also. It is noteworthy that E. J. Russell (17) found end products of protein hydrolysis in corn silage made in the presence of toluene, which hydrolysis he ascribed to enzym action.

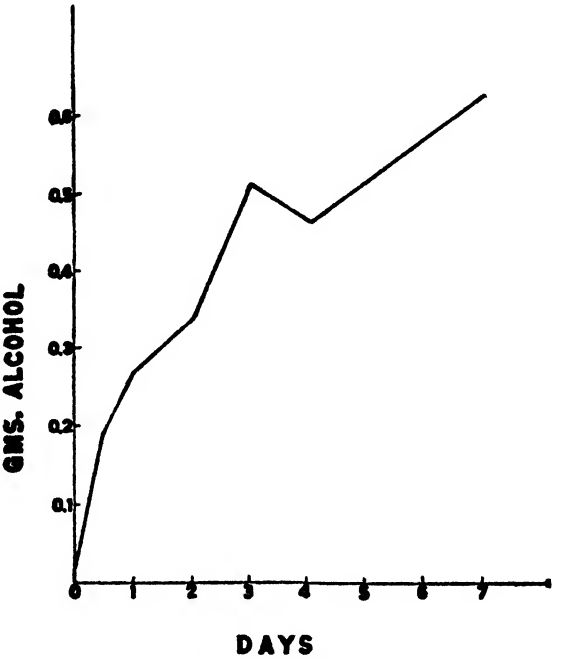

FIG. 12.—Curve showing the formation of alcohol in series 5.

The evolution of carbon dioxid must be due largely to enzym action. The curves shown all agree on that point, even when the first period was only 12 hours. Evidence that yeasts produce a part of the carbon dioxid after the first day has also been pointed out.

The rise in temperature of the silage is not great except at the surface, where the material is in contact with air. Microorganisms seem to be responsible for most of the heating, but the partial influence of enzyms is not excluded.

SUMMARY

The question of the respective causal relationship of microorganisms and plant cell enzyms to the fermentation of corn silage has long been in controversy. It is difficult to differentiate between the activities of these two kinds of agents. Work with antiseptics both by earlier investigators and by the writer is not conclusive. Experimental silage, other

than antiseptic silage, has been made, with results of some value; but the most conclusive evidence is obtained by the determination of the rate of change in various phenomena of the fermentation under normal conditions. Curves plotted from these data show that bacteria are mainly responsible for acid production and the concomitant disappearance of sugars. Alcohol is formed first by plant enzyms and later by yeasts. Protein is hydrolyzed first by enzyms and later by microorganisms. Carbon dioxid evolution seems to be very largely due to respiratory or enzymic activities, but yeasts probably have a share in its production after the first day or two. Microorganisms are probably largely responsible for the heating of the silage. Both kinds of factors are always present during silage fermentation and the process is due to the activities of both in the absence of air.

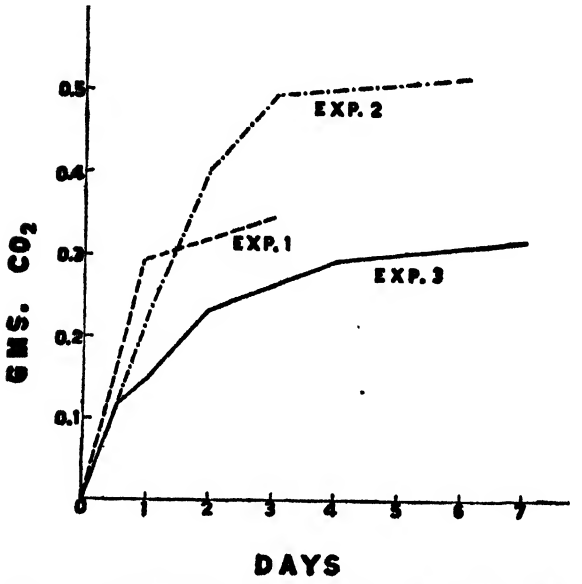

FIG. 13.—Curves showing the rate of evolution of carbon dioxid. (Curves 2 and 3 are coincident during the first one-half day.)

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LITTLE-LEAF OF THE VINE

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OCCURRENCE OF THE DISEASE

A disease of the vine (*Vitis* spp.) known variously as "little-leaf," "curly-leaf," and "yellow-leaf" first attracted attention in California about the beginning of the century. Some growers claim to have noticed it earlier, but no printed reference has been found that points clearly to this disease before 1900. It seems probable that it was present earlier, but most of the vines in many districts where it is prevalent have been planted since that date. This may account for the apparent spread and increase of the disease during the last 10 or 15 years.

At present, many vines are attacked in various regions, from the borders of Sacramento and San Joaquin Counties to the southerly end of the San Joaquin Valley. No indubitable cases have been noted in the Sacramento Valley, south of Kern County, or in any part of the coast region. The total area attacked is difficult to estimate, but it is large. In some localities, only occasional vines or small spots are affected; in others, most of the vines show more or less intense symptoms.

LOSSES

The loss in crop is also large and equally difficult to estimate. In some regions the affected spots are fairly definite, the vines badly injured, and the crop almost a total failure. These regions, however, represent the smallest part of the loss. Where the disease attacks the vines with great severity, if the affected spots are large, the whole vineyard is usually abandoned. It is only where the diseased spots are small that they are allowed to remain. The greatest loss occurs in the vineyards where the disease appears in a milder form and is distributed over wide areas. In such cases the crop may be diminished one-half, one-third, or less. The total loss of crop for California from little-leaf is considered by some observers to be greater than that due to any other vine disease. This is probably an exaggeration; but undoubtedly it takes rank with mildew, vine hoppers, and phylloxera as one of the most destructive.

GENERAL CHARACTER OF THE DISEASE

A vine, when very slightly affected, shows nothing but a few lightish areas or broad stripes on some of the larger leaves near the middle of the cane. These are not very noticeable and appear to be due to a slight deficiency of chlorophyll in the parts affected. On such vines the crop

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may be nearly normal, but there are usually many abortive berries ("shot grapes"). Vines badly affected show small yellowish leaves, short joints, upright canes, and abnormally numerous laterals (Pl. 89, *A*). The blossoms on such vines may all drop without setting, or a few normal berries may form on some of the bunches. Such vines seldom yield more than half a normal crop. Very badly affected vines show the same symptoms in an intensified degree, and toward the end of summer many of the leaves turn brown in whole or in spots. These vines bear nothing, and usually die after a year or two.

The appearance of a badly affected vine is very distinct, and the symptoms are unmistakable. They suggest very strongly a specific disease; but no bacterial, fungus, or animal parasite has been found in connection with them.

RELATION TO ENVIRONMENT

DISTRIBUTION IN THE VINEYARD

The distribution of the diseased vines is of two general types, which may be called the "diffusive" and the "intensive."

The diffusive type occurs usually in the lightest sandy soils. Here every vine in the vineyard may show symptoms. The disease in this case is seldom or never severe enough to kill the vines, but may seriously diminish the crop. It varies with the year and the variety. Some varieties may be almost immune, and in some years very few vines show the effects of the disease.

The intensive type occurs usually in sandy loams. In some cases of this type, a few badly diseased vines, even a single one, may be found completely surrounded by apparently perfectly healthy vines. Generally the diseased vines occur in irregular, ill-defined spots. Most of the badly diseased vines are found more or less in groups, but some of them are found among the surrounding healthy vines and sometimes a healthy vine will be found surrounded by a group of badly diseased. Healthy vines are seldom found near the middle of a large group of badly diseased vines.

Plate 92, *B*, and text figure 1 illustrate the intensive type of distribution. Figure 1, *A*, represents a vineyard where the attack is moderate and which is still producing paying crops. There is no evidence that the disease has killed any vines, as the number of vacancies (2.1 per cent) is quite normal and they occur among healthy and diseased vines with about equal frequency. Only 17.9 per cent of the vines are so badly diseased that they produce practically no crop; 26.6 per cent are noticeably affected and produce, on the average, about one-half of a normal crop. The remainder, 53.4 per cent, are apparently healthy. Such a vineyard would produce probably about two-thirds of a normal crop.

Figure 1, *B*, represents a very bad case of the disease. The vacancies represent 20.3 per cent of the area, and they are most frequent among

the worst vines. It is evident that the disease has killed many. Badly diseased vines represent 43.7 per cent, slightly diseased 13.7 per cent, and healthy vines 22.3 per cent. Such a vineyard would yield only about one-fourth of a normal crop.

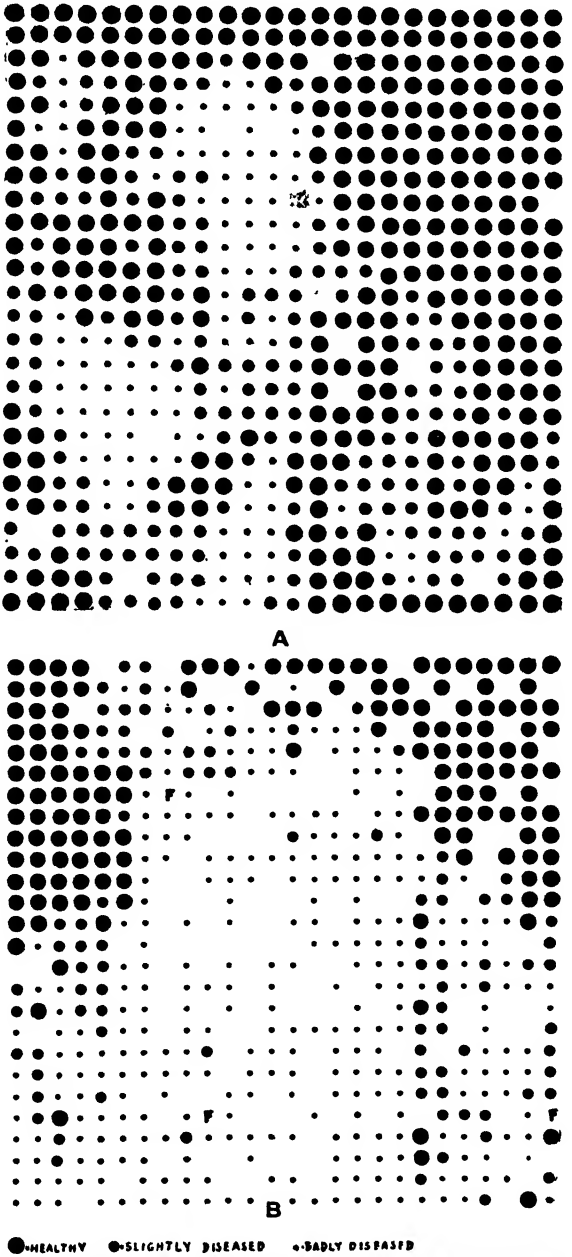


FIG. 1.—Diagrams of the distribution of grapevines affected with little-leaf: *A*, Moderately affected vineyard; *B*, a very badly affected vineyard.

CHARACTER OF THE SOIL

Typical or serious cases of little-leaf have been noted only in sandy or sandy-loam soils. If one passes through an infected region, the disappearance of the disease with a change from sandy to clay soil is very noticeable. The few cases in which the symptoms of little-leaf have been noted on vines growing in heavy soils do not seem to be typical and can probably be attributed to the soil of old sheep camps, which affects both vines and fruit trees in a similar way.

Perfectly healthy vines are found growing in sandy soil which on superficial inspection seems to be identical with adjoining soil where the vines are diseased. The soils in cases of this kind were examined in the attempt to determine whether any chemical or physical differences could be found which might account for the disease.

Sample I was taken from a soil on which all the vines were healthy. Samples II and III were obtained from samples of soil taken from the same vineyard about 300 yards from Sample I. Sample II was taken from between healthy vines, and Sample III from between diseased vines.

The chemical analyses of these three soils show such similarity of composition that they throw no light on the cause of the disease (Table I).

TABLE I.—*Chemical composition of vineyard soils (W)*

[Analysis made by the Division of Soil Bacteriology, University of California.]

Constituent	Sample I (soil bearing healthy vines)		Sample II (soil bearing healthy vines).		Sample III (soil bearing diseased vines).	
	1 ft	2–4 ft.	1 ft.	2–4 ft.	1 ft.	2–4 ft.
Insoluble matter	72.98	70.75	75.87	675.21	074.26	74.77
Soluble silica	10.02	11.40	8.03	8.10	8.86	9.18
Potash (K_2O)	.80	.83	.47	.51	.59	.59
Soda (Na_2O)	.20	.35	.26	.23	.23	.24
Lime (CaO)	1.25	1.22	1.16	1.21	1.11	1.11
Magnesia (MgO)	1.11	1.15	.88	.91	.88	.93
Brown oxid of manganese (Mn_3O_4)	.11	.07	.08	.06	.05	.05
Peroxid of iron (Fe_2O_3)	4.38	4.88	5.94	6.15	5.50	5.60
Alumina (Al_2O_3)	5.96	6.97	5.29	5.85	5.55	5.73
Phosphoric acid (P_2O_5)	.19	.14	.18	.13	.12	.10
Sulphuric acid (SO_3)	.05	.21	.05	.04	.05	.05
Water and organic matter	3.20	2.47	2.16	1.91	2.97	1.90
Total	100.25	100.44	100.37	100.31	100.17	100.25
Humus nitrogen in soil, per cent	.073	.042	.067	.037	.064	.027

An examination of the physical constitution of the soil showed something more definite (Table II). Two samples were taken in each of several vineyards. One of these samples (marked "D") was taken from a spot where the vines were badly diseased, the other (marked "H") from a spot where the vines showed no symptoms of disease. In all cases sample D and its companion, sample H, were taken from spots as near together as possible and where there was no visible difference in color, texture, or situation.

TABLE II.—*Percentages of coarse material and of water in soil*[a]

W. VINEYARD (DIFFUSIVE TYPE OF ATTACK; NOT VERY SEVERE)

Date.	Depth.	Coarse material.			Moisture.		
		D	H	D–H	D_1	H_1	D_1+H_1
1912.	*Feet.*						
July	1	24.93	22.56	2.37	4.33	6.39	.....
Do	2	37.15	25.60	11.55	5.49	7.22	.....
Do	3	27.23	25.03	2.20	7.86	10.20	.....
Means	.....	29.77	24.39	5.38	5.89	7.93	74.2

[a] Determinations made by Mr. L. Bonnet.

TABLE II.—*Percentages of coarse material and of water in soil*—Continued

M. VINEYARD (DIFFUSIVE TYPE OF ATTACK; SEVERE. SOIL VERY LIGHT AND SANDY)

Date.	Depth.	Coarse material.			Moisture.		
		D	H	D−H	D1	H1	D1+H1
1912	*Feet.*						
July	1	59.60	43.09	16.51	2.86	0.88	
Do	2	67.38	39.21	28.17	4.84	1.68	
Do	A 3	72.98	44.86	28.12	4.77	1.71	
Do	4	75.18	40.89	34.29	4.38	1.98	
Do	1	66.00	51.30	14.70	3.88	2.19	
Do	2	72.13	48.93	23.20	4.66	3.87	
Do	B 3	63.81	52.45	11.36	3.83	3.28	
Do	4	62.40	52.55	9.85	3.89	3.77	
Do	1	52.04	45.63	6.41	3.79	3.34	
Do	2	56.25	51.06	5.16	4.85	4.43	
Do	C 3	60.70	49.81	10.89	4.76	2.60	
Do	4	57.65	49.36	8.29	4.50	2.36	
Means	....	63.84	47.43	16.41	4.25	2.67	159

E. VINEYARD (INTENSIVE TYPE; VERY FEW VINES ATTACKED, BUT THESE SEVERELY. LOAM SOIL, RICH.)

1912							
July	1	18.05	16.86	1.19	1.64	1.33	
Do	2	21.02	17.68	3.34	4.42	3.26	
Do	3	19.80	18.58	1.22	7.00	5.49	
Do	4	18.75	20.00	−1.25	5.11	4.47	
Do	5	23.20	17.00	6.20	5.61	3.93	
Do	6	26.71	17.40	9.31	5.50	5.48	
Means		21.26	17.92	3.34	4.88	3.99	122.3

S. VINEYARD (TYPE OF ATTACK INTERMEDIATE BETWEEN THE INTENSIVE AND THE DIFFUSIVE MORE SEVERE THAN W., LESS THAN M.)

1912							
July	1	51.31	27.21	24.10	5.99	7.73	
Do	2	35.38	33.25	2.13	5.00	9.83	
Do	3	42.17	32.23	9.94	8.21	12.30	
Do	4	48.80	25.80	23.00	11.10	14.58	
Do	1	27.95	29.88	−1.93	5.15	6.17	
Do	2	39.02	31.88	7.14	6.70	6.75	
Do	3	45.55	33.25	12.30	8.43	11.81	
Do	4	41.90	27.53	14.37	11.44	11.82	
Do	1	35.74	22.78	12.96	4.34	6.08	
Do	2	37.48	26.81	10.67	5.65	6.85	
Do	3	39.94	31.45	8.49	9.16	8.91	
Do	4	35.45	30.45	5.06	11.10	12.07	
Means	..	40.00	29.38	10.68	7.61	9.58	79.4
August	1	34.06	22.13	11.93	3.70	6.00	
Do	2	41.92	24.01	17.91	4.69	7.68	
Do	3	45.33	25.36	19.97	4.50	10.54	
Do	4	50.05	12.57	37.48	7.02	19.17	
Do	1	33.25	28.05	5.20	2.89	5.33	
Do	2	30.80	26.34	4.40	4.63	6.11	
Do	3	40.60	30.40	10.20	5.50	8.32	
Do	4	51.50	31.10	20.40	7.44	10.58	
Do	1	30.66	23.89	6.77	3.53	4.85	
Do	2	39.75	27.20	12.55	4.52	5.40	
Do	3	38.43	27.86	10.57	4.67	7.14	
Do	4	41.00	30.86	10.14	5.91	9.20	
Means	.....	39.78	25.81	13.97	4.91	8.36	58.7
September	1	34.48	25.35	9.13	3.38	6.05	
Do	2	38.33	24.38	13.95	3.66	6.62	
Do	3	39.00	25.00	14.00	4.26	10.85	
Do	4	44.28	22.10	22.18	5.48	13.95	
Do	1	33.26	26.13	7.13	3.17	5.12	
Do	2	34.93	27.90	7.03	3.69	5.09	
Do	3	39.86	29.20	10.66	4.54	6.53	
Do	4	35.70	30.95	4.75	5.70	8.95	
Do	1	33.06	22.28	10.78	2.38	3.62	
Do	2	33.41	22.10	11.31	2.66	4.19	
Do	3	33.48	24.95	13.53	2.95	5.38	
Do	4	36.05	30.98	5.07	3.60	8.30	
Means	.....	36.73	25.94	10.79	3.79	7.05	53.8

TABLE II.—*Percentages of coarse material and of water in soil*—Continued

S. VINEYARD (TYPE OF ATTACK INTERMEDIATE BETWEEN THE INTENSIVE AND THE DIFFUSIVE. MORE SEVERE THAN W., LESS THAN M.)—continued

Date.	Depth.	Coarse material.			Moisture.		
		D	H	D−H	D1	H1	D1+H1
1912	*Feet.*						
October	1	31.55	21.93	9.62	3.18	5.22	.....
Do.	2	29.08	27.95	1.13	3.55	5.70	.....
Do.	3	39.60	28.28	11.32	4.52	6.84	.....
Do.	4	47.08	24.93	22.15	5.21	7.86	.....
Do.	1	29.38	25.88	3.50	3.08	3.72	.....
Do.	2	34.05	29.48	4.57	3.65	5.12	.....
Do.	3	38.30	25.57	12.73	4.52	6.04	.....
Do.	4	42.65	29.90	12.75	5.27	7.72	.....
Do.	1	33.22	23.05	10.17	2.24	3.98	.....
Do.	2	34.30	21.25	13.05	2.38	4.54	.....
Do.	3	34.73	26.23	8.50	2.54	5.26	.....
Do.	4	35.15	26.23	8.92	3.21	8.86	.....
Means	.....	35.76	25.89	9.87	3.61	5.91	61.1
November	1	29.56	22.75	6.81	3.56	6.71	.....
Do.	2	37.58	25.88	11.70	3.49	7.43	.....
Do.	3	39.23	25.13	14.10	4.15	9.13	.....
Do.	4	40.05	19.28	20.77	4.61	12.52	.....
Do.	1	20.50	24.00	−3.50	3.62	5.34	.....
Do.	2	33.00	28.06	4.94	3.58	5.66	.....
Do.	3	32.08	27.00	5.08	5.91	6.56	.....
Do.	4	42.70	27.68	15.02	3.05	8.40	.....
Do.	1	31.30	20.07	11.23	3.70	5.02	.....
Do.	2	34.55	22.33	12.22	3.18	5.10	.....
Do.	3	43.20	25.48	17.72	3.40	5.71	.....
Do.	4	39.00	26.32	12.68	4.14	7.75	.....
Means	.....	35.23	24.50	10.73	4.03	7.11	56.7
1913							
March	1	28.98	20.90	8.08	3.24	5.91	.....
Do.	2	33.28	22.27	11.01	3.24	7.57	.....
Do.	3	35.00	20.64	14.36	3.63	8.32	.....
Do.	4	39.38	20.22	19.16	4.56	10.61	.....
Do.	1	26.26	24.22	2.04	3.80	4.65	.....
Do.	2	28.63	26.46	2.17	4.10	5.60	.....
Do.	3	30.10	25.27	4.83	4.44	6.63	.....
Do.	4	40.50	26.12	14.38	5.27	8.32	.....
Do.	1	29.30	22.15	7.15	3.71	5.17	.....
Do.	2	31.30	22.38	8.92	3.81	5.42	.....
Do.	3	33.43	24.38	9.05	4.05	5.97	.....
Do.	4	33.70	24.20	8.50	4.46	7.45	.....
Means	.....	32.49	23.27	9.22	4.03	6.80	59.3
April	1	32.10	19.88	12.22	2.96	5.80	.....
Do.	2	35.58	18.71	16.87	3.31	7.31	.....
Do.	3	39.23	23.72	15.51	3.91	10.24	.....
Do.	4	43.98	22.90	21.08	5.23	11.62	.....
Do.	1	28.10	23.68	4.42	3.18	4.26	.....
Do.	2	33.95	23.76	10.19	3.87	5.68	.....
Do.	3	43.15	28.68	14.47	5.14	6.92	.....
Do.	4	43.50	27.90	15.60	5.99	9.30	.....
Do.	1	31.22	26.48	4.74	3.12	4.09	.....
Do.	2	32.32	21.10	11.22	3.72	5.37	.....
Do.	3	34.18	23.12	11.06	4.02	6.42	.....
Do.	4	38.10	28.02	10.08	4.78	8.61	.....
Means	.....	36.28	24.00	12.28	4.10	7.14	57.4
May	1	28.55	21.75	6.80	3.00	6.61	.....
Do.	2	35.48	24.07	11.41	3.93	8.26	.....
Do.	3	33.86	22.06	11.80	4.84	10.09	.....
Do.	4	38.85	21.65	17.20	6.63	13.35	.....
Do.	1	29.86	23.48	6.38	3.87	4.96	.....
Do.	2	27.97	26.56	1.41	4.13	6.35	.....
Do.	3	34.41	30.75	3.66	5.08	7.62	.....
Do.	4	40.75	33.13	7.62	7.55	9.74	.....
Do.	1	27.13	23.90	3.23	3.74	5.53	.....
Do.	2	32.72	24.85	7.87	5.83	6.22	.....
Do.	3	43.38	25.88	17.50	5.02	6.91	.....
Do.	4	44.38	27.20	17.18	6.41	9.46	.....
Means	.....	34.78	25.44	9.34	4.84	7.92	61.1

TABLE II.—*Percentages of coarse material and of water in soil*—Continued

8. VINEYARD (TYPE OF ATTACK INTERMEDIATE BETWEEN THE INTENSIVE AND THE DIFFUSIVE. MORE SEVERE THAN W., LESS THAN M.)—continued

Date.	Depth.	Coarse material.			Moisture.		
		D	H	D−H	D1	H1	D1+H1
1913	*Feet.*						
June	1	34.04	21.65	12.39	2.00	7.20	.....
Do	2	33.05	21.45	11.60	3.87	8.72	.....
Do	3	38.39	24.08	14.31	5.47	11.08	.....
Do	4	40.65	18.15	22.50	7.76	12.56	.....
Do	1	28.22	23.76	4.46	3.36	4.90	.....
Do	2	33.90	24.43	9.47	4.48	6.46	.....
Do	3	36.36	29.00	7.36	6.11	8.82	.....
Do	4	35.03	27.55	7.48	8.38	12.37	.....
Do	1	29.02	22.22	6.80	2.87	4.67	.....
Do	2	31.52	22.85	8.67	4.08	5.94	.....
Do	3	33.07	23.50	9.57	5.75	8.63	.....
Do	4	38.83	28.42	10.41	7.36	11.60	.....
Means	.. ..	34.34	23.92	10.42	5.12	8.58	59.7

In every case where the vines were diseased the soil shows more coarse material than the companion soil, where the vines were healthy. This is true in every vineyard examined and at all depths.[a] This uniformity indicates strongly a connection between the disease and the coarseness of the soil. That the coarse material is not alone responsible is shown by the fact that in the W. vineyard diseased vines were found where the proportion of coarse material was only 29.77 per cent, while in the M. vineyard healthy vines were growing in soil containing 47.43 per cent. It is to be noted, however, that the intensity of the disease is in proportion to the coarseness of the soil, both when we compare soils from different vineyards and where we compare soils from different parts of the same vineyard. The difference between the coarse material in the diseased parts of the vineyard and in the healthy parts (D − H, Table III) is roughly a measure of the intensity of the disease. Vineyard K. is a special case. The difference D − H is small, but the diseased vines were very badly affected. There were, however, but a few single vines or groups of two or three vines affected and all the rest appeared perfectly healthy.

TABLE III.—*Percentage of coarse material in diseased and in healthy portions of vineyard (summarized from Table II)*

Vineyard and character of attack.	Percentage of coarse material.		
	Diseased.	Healthy.	D−H.
M. Attack diffusive; severe	63.84	47.43	16.41
S. Attack intermediate; moderately severe	36.16	25.35	10.81
W. Attack diffusive; not severe	29.77	24.39	5.38
K. Attack intensive; very few vines affected, but these badly	21.26	17.92	3.34

[a] Of 129 pairs of samples examined, there were only 3 exceptions, and these apply to only a single foot and not to the whole soil depth.

The differences noted in the moisture contents of diseased and healthy spots were less concordant, as shown by Table IV.

TABLE IV.—*Moisture in diseased and in healthy portions of vineyard (from Table II)*

Vineyard and character of attack.	Percentage of moisture.		
	Diseased.	Healthy.	D+H.
M. Attack diffusive; severe	4.25	2.67	159.0
S. Attack intermediate; moderately severe	4.62	7.61	60.7
W. Attack diffusive; not severe	5.89	7.93	74.2
K. Attack intensive; very few vines affected, but these badly	4.88	3.99	122.3

In the S. and W. vineyards the moisture contents were notably lower in the diseased spots than in the healthy, but in the other vineyards they were higher in about the same proportion. The most weight should be given to the results in vineyard S. on account of the large number (108) of determinations made. In every case in this vineyard, at all depths, the soil in diseased spots was drier than in healthy spots. The same is true of the W. vineyard. In the M. and the K. vineyards this condition is reversed. In M. the soil is excessively sandy, of the type known as "blow sand," and in K. the soil is the least sandy of all. Both these vineyards were irrigated, and the moisture conditions observed may not be typical of the whole year. The S. vineyard was not irrigated, and the observations cover the greater part of the year, from March to November. It seems fair to conclude, therefore, that a lack of moisture is a characteristic of affected soils.

ANNUAL AND SEASONAL VARIATIONS

The disease does not seem to be progressive—that is, it does not necessarily increase with succeeding years. A vineyard may be sufficiently affected one year to lose most of its crop and show practically no sign of the disease the next. The trouble returns, however, in subsequent years. In most affected vineyards it is noticeable every year, but with varying degrees of intensity. Very badly diseased vines seem never to recover, and they finally die. The annual variation in a moderately affected vineyard (St. vineyard) and in a badly affected vineyard (F. vineyard) are shown in Table V.

TABLE V.—*Percentage of annual variations of attack in the St. and F. vineyards*

Condition.	St. vineyard.		F. vineyard.			
	1905	1906	1910	1911	1912	1913
Vines dead or missing	0	0	9.7	[a] 2.5	2.5	3.2
Vines badly affected	0	10.5	56.8	29.9	33.3	40.1
Vines slightly affected	20.5	17.7	29.9	34.9	21.2	18.7
Vines healthy	79.5	71.8	12.6	38.7	43.0	38.1

[a] Missing vines of 1910 replaced with new plantings.

The St. vineyard consisted of old Tokay vines; the F. vineyard of young (5 years old in 1910) Zinfandel vines grafted on Rupestris St. George. In the F. vineyard, there was marked improvement in 1911, and a slight relapse during subsequent years.

The sequence of the symptoms during the year is peculiar. All vines, except those very badly affected, appear perfectly healthy during the early spring. It is only when the weather becomes warm, usually in May, June, or July, that the symptoms become marked. They increase until about the commencement of the vintage. At this time they commence to disappear and moderately affected vines may appear perfectly healthy in October and November. The healthy character of the first growth is indicated in Plate 89, *B*. The leaves on the lower part of the canes appear quite healthy. These are the first leaves formed in the spring. The disease became noticeable about the time of the appearance of the blossoms; and the result is shown in the small, abortive berries and the small leaves on the upper parts of the canes.

SYMPTOMS OF LITTLE-LEAF

LEAVES

Slightly affected leaves are of normal size, or nearly so, but show a tendency to curl up at the edges. They show bands or patches of light-colored parenchyma. The rest of the leaf appears somewhat darker than normal, but this may be the effect of contrast. Such leaves appear on very slightly affected vines or at the bases of canes more badly affected (Pl. 90, *B*).

More badly affected leaves are smaller and the bands of light colored parenchyma are larger and more numerous. These bands are not regular nor distinctly marked, but cause the general color of the foliage of a diseased vine to be lighter than that of a healthy one. Such leaves occur chiefly on the middle portions of the canes.

In very badly affected leaves these symptoms are intensified, and the light yellowish bands or spots dry, turn brown, and crack. When the canes are shaken, the leaves make a sound like dry leaves. They are so brittle that when folded they crack. The petioles are abnormally short and thick.

The microscopical examination of sections showed very little difference except a scarcity of chlorophyll grains in the diseased leaves. Though these feel thicker, micrometric observations showed that they were actually thinner than the corresponding healthy leaves. Ninety-three measurements of healthy and diseased leaves gave an average thickness of 194.3 μ for the former and 170.2 μ for the latter. These measurements were made on Feher Szagos and Zinfandel.

The shape of the leaf is not altered. A large number of measurements showed no change in the ratio of length to width.

The difference in size varied with the intensity of the disease, with the position of the leaf on the cane, and with the variety (Table VI).

TABLE VI.—*Comparison of size of healthy and diseased leaves of the vine*

Order of leaf on cane.	(Length of healthy leaf)÷(Length of diseased leaf).		
	Petite Sirah.	First Zinfandel.	Second Zinfandel.
1	.....	.....	.....
2	.....	1.11	1.56
3	0.81	.....	.....
4	.....	1.19	1.56
5	1.17	1.04	2.00
6	1.26	1.64	2.86
7	1.27	.....	.....
8	1.53	1.60	3.03
9	1.42	1.94	4.38
10	1.50	2.45	6.64
11	1.77	3.53	.....
12	.....	3.12	.....
Means	1.34	2.00	3.14

This table shows three degrees of intensity of attack. With the Petite Sirah there was little difference in size of leaves up to the fifth from the base of the cane. From this point the leaves on the diseased vines gradually became smaller, until at the eleventh node the diseased leaf was only 56 per cent as long as the corresponding healthy leaf. The first Zinfandel shows an increase of intensity. The lower leaves up to the fifth showed little difference, but the eleventh on the diseased vine was only 32 per cent as long as the healthy leaf. The second Zinfandel represents a very severe case. Even the lowest leaves were much smaller (64 per cent), and the tenth leaf of the diseased vine was only 15 per cent as long as the healthy leaf.

These measurements were made early in May and do not show the decrease of intensity shown later by the production of almost normal leaves.

CANES

As with the leaves, the symptoms on the shoots and canes vary with the intensity of the disease. The lower or earlier internodes may be nearly or quite normal, except in very severe cases. From the middle, and especially on the laterals, the internodes are shortened and flattened.

The shoots and canes are very erect. This, with the abnormal number of laterals, gives the vine a bushy appearance, completely changing its natural habit. In a vineyard of moderately affected vines the average length of the shoots of diseased vines was found to be 49 per cent of that of the shoots of healthy vines. This corresponds to the average difference between the lengths of the internodes, which was in the ratio of 100 to 50.

The flattening, or ratio between the longest and shortest diameters of the canes, is marked in some cases. Measurements made showed this ratio to average 1.22 in healthy vines and 1.38 in corresponding diseased vines.

ARMS

On the 2-year-old wood, from which the canes arise, the flattening is even more common and pronounced. The arms on badly diseased vines are shorter and less numerous than on healthy vines. This is due to the dying back of the second- and third-year-old wood and the removal of the arms in pruning. The water sprouts from old wood being usually larger and more vigorous than the fruit canes on younger wood, the pruner has a tendency to utilize them for spurs. This tends to keep the arms short.

A section through the 2-year-old part of an arm shows yellowish points usually confined to the 2-year-old wood. Sometimes these points are so numerous as to color the whole surface of the section and then a few points may occur on the 1-year-old wood. These yellow points occur also on apparently healthy arms, but rarely and never abundantly.

Sections made successively through older parts of the arm show more numerous and darker points. In wood 4 to 6 years old these points are nearly black. They are usually scattered through all annual growth rings except that of the current year.

The microscope reveals in these dark and yellowish spots the presence of thyloses and gum in the xylem tubes, parenchymatous cells and in a few xylem fibers adjoining them (Pl. 90, *A*, 91, *A*, *B*). The medullary rays close to affected tissues show dark cells filled with a granular and brown substance unstained by iodin.

TRUNK

Dark points like those in the arms are also found in the trunk. They can be followed almost to the base of the underground stem. At the head of the vine, where the arms start, occur the most abundant diseased areas. These gradually diminish in number as we approach the base of the underground stem. This progressive diminution is shown in Plate 91, *C*–*E*. This represents three sections through the trunk of a badly affected 3-year-old Petite Sirah vine. Figure *C* is a section at the branching point of the arms about 12 inches above the surface of the ground. The wood of the first two years is all of a uniform yellowish brown color. *D* is a section at the level of the ground. It shows 19 dark dots or small patches distributed through 2- and 3-year-old wood. *E* was made at 6 inches below the surface and shows only 9 of these dots. Only 3 dots were found in a section made at the base of the underground stem.

Microscopically, these diseased areas show the same gummy matters noted in other parts, but they are darker. When the tissues are stained

with carbol-fuchsin, the xylem fibers stain red and the xylem tubes orange, indicating an abnormal condition of the walls of the xylem tubes.

ROOTS

The diseased spots may be found in the large roots, which when split show dark longitudinal lines corresponding to these spots.

FRUIT

The effect on the crop has already been noted. On slightly affected vines the crop may be diminished considerably. Those at all badly affected have a tendency to fail to set their fruit, and much of that which does set fails to develop or mature.

A microscopical examination of the pedicels of diseased bunches shows that there is little activity in the cambium. A lack of thickening of the pericylic fibers and of the collenchyma is also noted which explains the fragility of the stems and failure of the berries to develop (Pl. 91, *F*, *G*).

INFECTIOUSNESS

There is little or nothing in the distribution of the disease to indicate an infectious nature. Some growers claim that the infected areas gradually enlarge, but they are probably misled by the annual variations already noted. It is also possible that vines, so slightly affected at first that the injury passes unnoticed, gradually weaken and show the effects more plainly later, thus giving the impression of a spread of the trouble.

In some vineyards the disease has existed in spots for years without spreading. Perfectly healthy vines may show symptoms of little-leaf in the nursery and yet develop into perfectly healthy vines when planted in another soil. On the other hand, vines which appear healthy in the nursery may show the disease the first year when planted in certain soils. In the latter case, however, the disease does not usually manifest itself until the third or fourth year.

ETIOLOGY

No connection has been found between the disease and any vegetable or animal organisms. Bacteria have been found in diseased tissues, but inoculations in various ways afforded no evidence that they have any connection with the disease.

Young vines showing the disease in affected nurseries have been planted in districts where little-leaf does not occur, and they have developed normally, showing no symptoms of the disease in the new location. On the other hand, healthy vines from an unaffected district may show the disease the first year after planting in an affected district. Both of these cases are exemplified by direct experiments made at Davis

and Fresno and by thousands of vines distributed by nurserymen. The evidence seems conclusive that the cause is local, whether it exists in soil, water, or weather conditions.

VARIATIONS IN SUSCEPTIBILITY

The disease has been noted on various species of Vitis, and none has proved immune. It has been found on the following phylloxera-resistant stocks: Riparia gloire de Montpellier, Rip. grand glabre, Rupestris St. George, Vitis aestivalis, V. Champini, V. Doaniana, Berl. × Rip. 157–11, Rip. × Rup. 101–14, 3306, 3309, Rip. × Cord. × Rup. 106–8. Sol. × Rip. 1615, 1616.

All varieties of *Vitis vinifera* are attacked, so far as noted; but a few appear to be peculiarly susceptible and a few others to have some considerable degree of resistance. The Mataro is so badly affected as to make a class by itself, although the Carignane is almost as bad.

Examples of partial resistance have been noted with Black Prince, Burger, Sultanina, Valdepeñas, Petite, and Alicante Bouschet. A vineyard of Mataro was found with all the vines nearly dead, except a few score scattering Black Prince vines which appeared perfectly healthy. Healthy Valdepeñas vines have been found in similar positions surrounded by badly affected vines of other varieties. Blocks of Sultanina, Burger, and the Bouschets, showing but negligible signs of disease, are growing adjacent to badly affected blocks of Carignane and other varieties. No badly affected vineyards or vines of these varieties have been found.

Badly affected vines in considerable quantities have been noted of Muscat, Tokay, Palomino, Feher Szagos, Zinfandel, Malaga, Green Hungarian, and Grenache.

Grafted vines seem to be as susceptible as vines on their own roots. The effect of grafting very susceptible or less susceptible varieties, and *vice versa*, has not been tried.

OTHER PLANTS AFFECTED

Several kinds of trees, differing very much botanically among themselves and from the vine, are affected by what seems to be the same trouble. This fortifies the idea that the disease is not infectious or parasitic. The location and distribution of the diseased trees are exactly parallel to those of the diseased vines, indicating a common cause. The Carolina poplar seems particularly susceptible, showing the small, pale leaves, abnormally numerous small branches, and generally stunted condition in a very marked degree. On affected poplars very remarkable examples of fasciation of the branches are common. Copious irrigation was noted in one case to increase the growth of these trees, but without apparently diminishing the other symptoms.

Apricot trees are also very badly affected with symptoms almost identical with those of the vine. Other trees, such as peach, walnut, almond, fig, and umbrella trees, show similar symptoms; but the resemblance to the trouble of the vine is less marked than in the cases of poplar and apricot. The symptoms have not been noted in any annual plant, even in the worst affected spots.

COMPARISON WITH OTHER VINE DISEASES

The symptoms distinguish little-leaf clearly from any other disease of the vine in California. To two European diseases, however, it shows a strong resemblance. These are "*mal nero*" and "*court noué*."

From the former it seems sufficiently distinguished by the different discoloration of the leaves. The "*mal nero*," moreover, is infectious and is not confined to special soils. The name "*court noué*," meaning short-noded, indicates one of the most characteristic symptoms of little-leaf. It appears to be applied, however, to several distinct diseases. One of these has been shown to be due to a minute arachnid attacking the leaves. Others have been traced to winter frosts and bacterial infections in the old wood. Others are unexplained and may be identical with our little-leaf.

CONTROL EXPERIMENTS

To seek to control a disease whose cause is unknown is to work in the dark. The need of a remedy, however, is so pressing that a considerable amount of work has been done in the hope of finding one.

INSECTICIDES AND FUNGICIDES

Affected vines were treated with various insecticides and fungicides on the supposition that the trouble might be due to some unperceived animal or vegetable parasite.

Vines were sprayed at the swelling of the buds, when the shoots were 6 inches long, and on the appearance of symptoms, with "Rex" and nicotine and with "Killthrips." No results were noticed.

Other vines were sprayed with Bordeaux mixture, iron sulphate, and dilute sulphuric acid in various combinations and at various times, but without any very noticeable results. Spraying in June with Bordeaux mixture produced some slight improvement for a few weeks, but later the treated vines were no better, if not worse, than the untreated. Other sprays had no noticeable effect.

Swabbing the vines when dormant with 5 and 10 per cent solutions of copper sulphate had no effect. Swabbing the pruning wounds with 0.5 and 0.1 per cent solutions of mercuric chlorid in winter resulted in the improvement of the vines early in the season, but later these vines looked worse than the untreated.

Injections of iron-sulphate solutions (1:500) and of copper-sulphate solutions (1:500 and 1:1,000) were made on a number of vines. The solutions were caused to enter the vine by means of a ½-inch hole bored halfway through the trunk near the surface of the ground. A metal tube was inserted into this hole and connected, by means of a rubber tube, to a funnel fastened to the head of the vine. The solution was poured into the funnel and was absorbed very readily. From 250 to 375 c. c. were used on each vine. These injections were made at various times from June 9 to September 10 in 1910. No results were obtained with the iron sulphate.

The copper sulphate produced some remarkable effects. One vine which received an injection of 375 c. c. of a 1 to 500 solution of copper sulphate on June 9 lost all its leaves in a few days, but a new growth started almost immediately, and by July 15 the vine was covered with a full supply of apparently healthy and normal foliage. A neighboring vine showing about the same degree of disease at the time of the injection was left untreated, and on July 15 had shown no signs of recovery (Pl. 92, *A*, *a*). All vines (11) which received injections of copper sulphate reacted in the same way. The first injury to the leaves and their subsequent recovery, however, were not so marked as with the first vine treated. The later the treatment, the smaller the effect. In all cases, however, the treated vines could readily be picked out by their more abundant, greener, and healthier looking foliage.

Examinations of the treated vines during the following year showed that the improvement had not been permanent. There was no apparent difference between the treated and the untreated vines when examined in 1911.

Similar injections were made with a larger number of vines during the following year in winter, spring, and in June. The winter injections apparently increased the disease. Those of spring and early summer had little effect, though some of those made in June improved the vines slightly. The vines treated this year were grafted, younger, less badly affected, and of a different variety.

CULTURAL MEASURES

The effect of fertilization was tried on about one-fifth of an acre in a patch of diseased vines. A complete fertilizer recommended by Prof. Burd was used at the rate of 400 pounds per acre. It had the following composition:

Superphosphate	P_2O_5 available	9.00	
Do	P_2O_5 insoluble	1.00	
			10.00
Sodium nitrate	N	1.50	
Ammonium sulphate	N	1.00	
Blood	N	2.50	
			5.00
Potassium sulphate	K_2O		4.00

Examinations made during the following seasons failed to show any difference in the amount of disease between the fertilized and the unfertilized portions of the patch.

It had been suggested by Mr. Frank Swett, of Martinez, and Prof. E. H. Twight that the trouble might be due to a deficiency of lime. Tests on nursery vines by Mr. Swett showed improvement in vines where lime had been used. Applications to old vines by Mr. Swett and others showed little or no effect. A test of the effect of gypsum was made by the California Agricultural Experiment Station on a small vineyard of 176 old Tokay vines, half of which was badly affected and half healthy or only

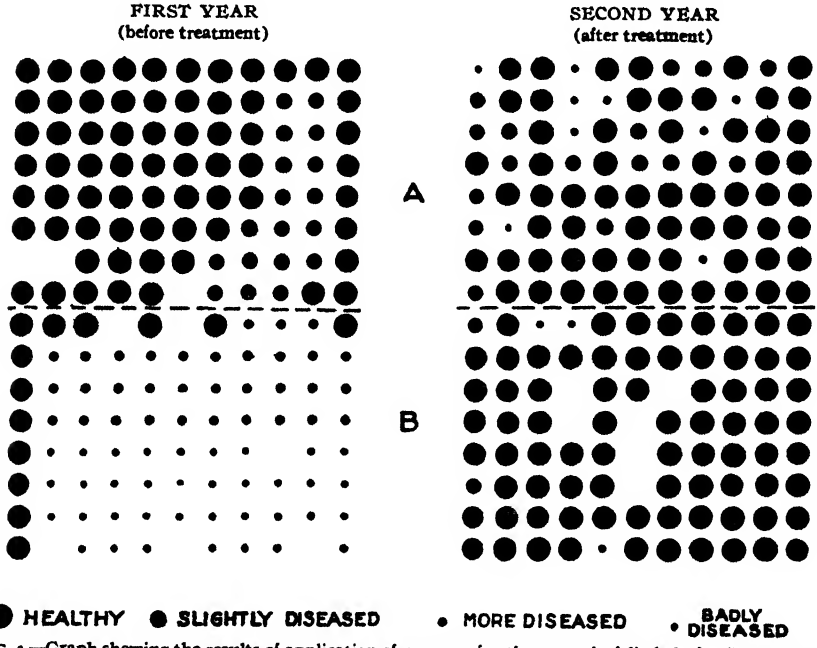


FIG. 2.—Graph showing the results of application of gypsum for the control of little-leaf. Gypsum was applied to the portion marked "B" during the winter of the first year. During the summer of the first year A was but slightly affected, B badly. During the summer of the second year A was a little worse, but B was almost free from disease symptoms.

slightly affected. The condition of the vines the year before the application was made and at a corresponding date of the year following the application is shown in figure 2 and Table VII.

The beneficial effect of the application of gypsum seems very apparent in this case. Block B, of which 83 per cent of the vines were diseased the year before the gypsum was applied, had only 5.8 per cent the following year—that is to say, the disease had almost disappeared. Block A, which had only 20.5 per cent of diseased vines the first year, had 29.6 per cent the second, showing that the improvement of block B was not due to the season. Unfortunately, this vineyard was close to a town, and was uprooted before further observations could be made.

TABLE VII.—*Results of application of gypsum to a vineyard for the control of little-leaf*

Block and condition.	First year.		Second year.	
	Vines.	Per cent.	Vines.	Per cent.
Block "A" (no treatment):				
Healthy	70	79.5	62	70.4
Slightly affected	18	20.5	16	18.2
More affected	.....	.....	.....	.....
Badly affected	.....	.....	10	11.4
Block "B" (1,000 pounds of gypsum per acre):				
Healthy	15	17.0	83	94.2
Slightly affected	.....	.....	2	2.3
More affected	33	37.5	.....	.....
Badly affected	40	45.5	3	3.5

Winter and early spring irrigation, so far as noted, seem to have little or no effect on the disease. The effect of summer irrigation has not been well tested.

Mr. A. Bonnet, of Montpellier, France, has suggested the plausible hypothesis that little-leaf is due to unfavorable changes of temperature, and specifically to a period of cold weather checking growth started by a previous period of warm weather. To test this hypothesis, measures were taken to delay the starting of the buds. This was accomplished by late pruning, whitewashing, and shading the vines. While a delay of nearly two weeks was obtained, no perceptible effect on the disease was noted.

SUMMARY

"Little-leaf" is a disease of the vine, vying in seriousness with oidium and phylloxera. It is confined to sandy soil in the great valley south of Sacramento County. It seems to be unknown elsewhere, unless a form of "*court noué*," noted in northern Mexico and southern Europe, are identical.

Affected vines show small, yellowish leaves, short-jointed canes, and in severe cases dead spots on the leaves and gummy secretions in the conducting tissues of the arms and trunks. In severe cases the vines die after a few years. Vines slightly affected set fruit imperfectly, and those badly affected bear little or nothing.

No parasite has been found connected with the disease, and the evidence seems to show that it is not infectious. Several species of trees are affected with similar symptoms when growing near or among affected vines, indicating that the disease is due to local conditions of soil, water, temperature, or other nonparasitic causes.

No effective method of treatment has been demonstrated, though the application of gypsum to the soil is promising.

PLATE 89

A.—A grapevine showing a severe infection of little-leaf.
B.—A moderately affected grapevine, showing healthy first leaves.

A

B

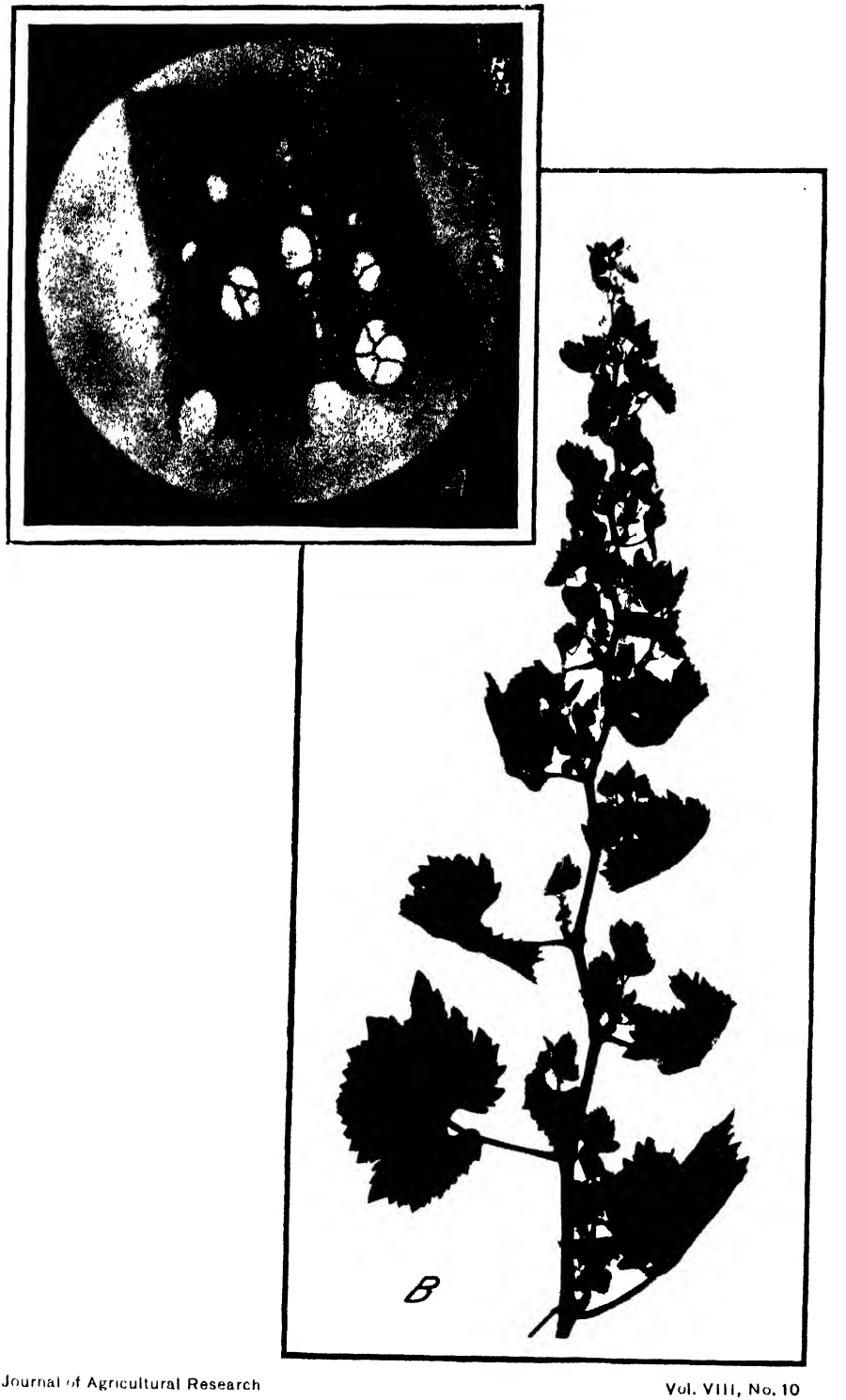
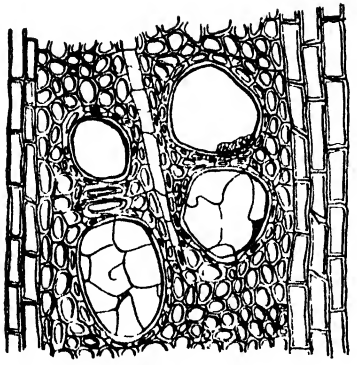

B

PLATE 90

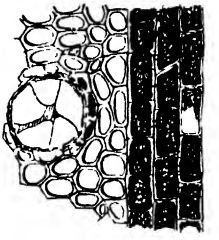
A.—Tyloses in the wood of the grapevine.
B.—Grapevine shoots showing little leaves and laterals.

PLATE 91

A, B.—Drawings of tyloses in the wood of the grapevine.
C, D, E.—Cross sections of the trunks of the grapevine.
F, G.—Cross sections of healthy and diseased pedicels of the grapevine.



A



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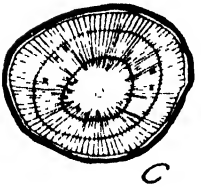

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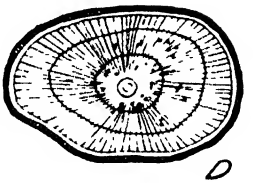

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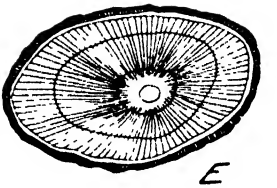

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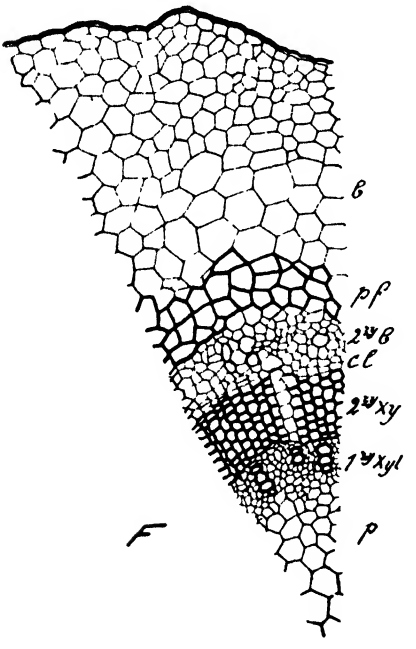

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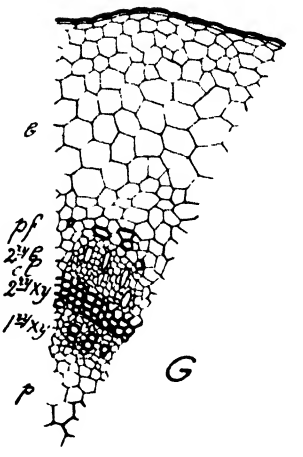
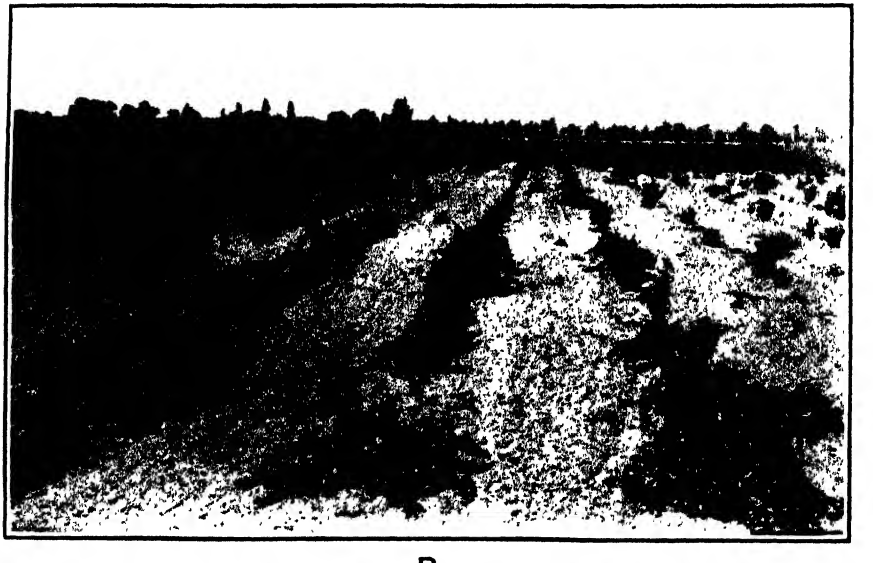


G

A



B

PLATE 92

A.—Grapevines showing the effect of treatment with an injection of copper sulphate: *a*, untreated; *b*, treated.

B.—Vineyard showing a severe case of the intensive form of little-leaf.

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SPORE-FORMING BACTERIA OF THE APIARY

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PART I.—DESCRIPTION AND COMPARISON OF SPECIES

INTRODUCTION

In a paper by Lambotte in 1902 (7)[1] the statement is made that "foulbrood" of bees can be produced by feeding the insects cultures of *Bacillus mesentericus vulgatus* grown upon a special medium prepared from the juices of bee larvæ. In explanation it is stated further that, by the use of this special medium, *B. alvei* arises as a special variety of *B. mesentericus vulgatus* and produces, on feeding, the tissue changes characteristic of the disease. Lambotte does not describe the disease adequately to make it possible to determine whether he worked with American foulbrood or European foulbrood, and at the time his paper appeared these two diseases were not differentiated clearly. This comparison of the two organisms disagreed so materially from the observations of the author that feeding experiments with *B. mesentericus vulgatus* were begun in 1911. At the outset it was observed that of the organisms of the *B. vulgatus* group, which are isolated in the course of the examination of specimens of bee comb and brood sent to the Bureau of Entomology for diagnosis, it was not possible to identify all as one species. Consequently, it was realized that the first task would be the identification of the various organisms of this group and a comparison of them, for purposes of easy identification, with the other known spore-forming bacteria of the apiary: *B. alvei*, *B. larvae*, and *B. orpheus*. Only *B. vulgatus*, *B. mesentericus*, and *B. orpheus* will be described in detail. For descriptions of *B. alvei* and *B. larvae* the reader is referred to White (16). The illustrations and summary at the end of the paper should aid in differentiating the five known spore-forming bacteria of the apiary. The results of the feeding experiments are given in Part II of this paper.

[1] Reference is made by number to "Literature cited," p. 419-420.

CULTURES STUDIED

SOURCE.—The cultures studied are obtained in the course of the daily examination of specimens of suspected brood and comb. Cultures can nearly always be obtained by adding bits of comb from any source to the melted agar of petri dishes at pouring. The organisms occur also variously distributed about the hive and can be secured from scrapings of hive covers, bottoms, and walls. They are also found occasionally in considerable numbers in some larvæ, as manifested by the goodly number of colonies occurring on agar plates after the contents of a broken-up larva have been added. Over 30 strains isolated from comb and brood from the various sections of the United States have been cultured and studied. For comparison, one culture of *B. vulgatus* was obtained from the Bureau of Animal Industry and one culture was isolated from potato.

AGE OF CULTURES STUDIED.—The cultures were studied at all ages, from the earliest date at which it is possible to isolate pure cultures and inoculate them into the various culture media up to over 4½ years. The rejuvenation of cultures after the method of Fuller and Johnson (4) was found valuable in obtaining constancy of cultural characteristics.

VARIATION.—From the foregoing statements concerning the age and number of cultures studied, it can be seen that abundant opportunity was afforded for observation, first, of the variation in the same strain due to environmental factors acting over a considerable period of time, and, second, of the variation as exhibited in a number of different strains of the same species. Variation due to both of these factors proved to be considerable, but there is no reason to believe that it is of any greater degree for these organisms than for many other bacterial species (1, 5, 6, 9, 11, 15).

Of the organisms of the *B. vulgatus* group isolated and studied, it seems best for the present to place all under two species only. Most of the organisms isolated are to be classed as *B. vulgatus;* a much smaller number belong to the species *B. mesentericus*. It is realized that further studies may show such great permanent differences in biology as to warrant a separation into still other species, especially of some of the organisms described as *B. vulgatus*. There is, however, one variety from sample of brood 2329[1] which presents such differences in cultural features that it is regarded as at least a special variety of *B. vulgatus*, if not a distinct species. This variety is described later in this paper.

MEDIA USED

REACTION.—All media are made 1.5 per cent acid to phenolphthalein, unless otherwise stated (3).

[1] In the original notes all cultures, as the above, are designated by a number, and this number refers to the sample sent to the Bureau of Entomology for diagnosis, from which the organism was originally isolated. By referring to the card on file for such sample, various data concerning the original source of the culture are available.

BOUILLON.—Most of the bouillon used was prepared from beef, and such bouillon is more satisfactory than that from meat extract, provided a good quality of fresh meat is available. Bouillon was also prepared from Liebig's meat extract, using 0.3 gm. of the extract per 1,000 c. c. of water, adding 1 per cent of Witte's peptonum siccum and 0.5 per cent of sodium chlorid. Bouillon prepared from meat extract should be used only when good fresh meat can not be secured.

SUGAR-FREE BOUILLON.—The small amount of muscle sugar present in bouillon made from meat is eliminated by inoculating with *B. coli communis*. Fermentation tubes of bouillon prepared from Liebig's meat extract, when inoculated with *B. coli communis*, failed to yield gas and increased acidity; hence, such bouillon may be used as a base for sugar media without being treated with this organism. Dunham's peptone solution is also used sometimes instead of sugar-free bouillon in growing cultures for the indol test and in preparing the various sugar media. Its use is quite satisfactory in the study of the reaction changes from day to day, but is not satisfactory for the observation of growth characteristics.

ACID AND NEUTRAL BOUILLON.—Plain bouillon is made neutral or acid to the desired degree by adding the proper quantity of *N*/1 sodium hydroxid or *N*/1 hydrochloric acid, as determined by titration.

AGAR.—One and one-fourth to one and one-half per cent of agar-agar in plain bouillon is used. The appearance on agar plates of the members of the group studied constitutes an important differential point; hence, this is a valuable medium. Owing to the absence of muscle sugar in the bouillon prepared from Liebig's extract, it was found desirable to add about 0.1 per cent of glucose to such agar medium.

POTATO SLOPES.—The potatoes are thoroughly washed and peeled by removing generously the outer portions, after which cylinders are removed by means of a cork borer and cut diagonally. The pieces are washed in several changes of water, tubed with a liberal quantity of water, and sterilized. It is advantageous to leave a liberal quantity of water in the tubes on inoculation, otherwise characteristic growth is not apt to occur.

SERUM.—This medium is prepared from both calf's blood and that of the horse by heating them over a period of several days in a serum inspissator after the usual described methods.

POTATO WATER.—A few of the strains were inoculated into potato juice prepared by adding an equal amount of water by weight to very small pieces of potato, boiling, straining, and filtering the mixture, and then distributing into test tubes. Little value is attributed to this medium alone, since the growth does not differ from that on slopes with a liberal quantity of water.

MILK.—Owing to the difficulty of procuring separator milk, bottled milk, as purchased on the market, is used, first pipetting off the top cream, heating, setting aside overnight, again pipetting off any layer of

cream, and repeating until milk with a minimum of cream fat is secured, after which it is distributed into straight tubes.

LITMUS MILK AND AZOLITMIN MILK.—In the earlier studies an aqueous solution of ordinary litmus was used; later it was found that a solution of the pure blue dye azolitmin eliminated the objectionable feature of the red dye, which is present when litmus solution, as ordinarily prepared, is used (14, p. 419).

GELATIN.—Bouillon is used as a base to which gelatin to the amount of 10 per cent is added. The character of the growth, rate of liquefaction, form assumed by the liquefied area, degree and character of turbidity, membrane, and sediment were noted.

GLYCERIN GELATIN.—To gelatin prepared as noted above 5 per cent glycerin was added. The growth was studied and noted as for plain gelatin.

REDUCTION OF NITRATES TO NITRITES.—In testing for nitrate reduction, cultures were grown in a medium made up of Witte's peptonum siccum, 1 gm., and sodium nitrate, 0.2 gm., in 1,000 c. c. of tap water. To the culture grown in this medium and to the control tube, after five days' incubation, there were added equal parts of solutions A and B of Griess's reagent for nitrites as follows:

Solution A (Sulphanilic acid): 0.5 gm. is dissolved in 150 c. c. of 3 per cent acetic acid.

Solution B (Alpha-naphthylamin): 0.1 gm. is boiled with 20 c. c. of water and filtered hot through a small filter from which nitrites have been removed by washing. It is then diluted with 180 c. c. of 3 per cent acetic acid.

INDOL.—Cultures are incubated for 10 days in either sugar-free bouillon or Dunham's peptone solution; at the end of this period, to the culture and to the control tube are added first strong sulphuric acid, carefully poured down the side of the tube, followed in the same manner by a 0.02 per cent solution of sodium or potassium nitrite. A delicate pink ring forms at the juncture of the acid and culture in the presence of indol, or a pink coloration of the whole tube on agitation, if the coloration of the ring is of sufficient intensity.

ADDITIONAL NOTES ON TECHNIC

MORPHOLOGY AND MICROMETRY.

(*a*) VEGETATIVE FORM.—In both length and breadth these two organisms, *B. vulgatus* especially, vary not a little. This variation occurs not only in different strains, but in the same strain at different times and on different media. It is not unlikely that a number of conditions operate to produce such variation, such as amount of moisture, temperature, amount and rate of growth, and various other undetermined factors of development. In giving measurements, data concerning the medium, age of culture, and staining should be noted. Twenty-four-hour-old agar cultures were used for vegetative forms, and owing to the

greater difficulty of getting good stained preparations of spores measurements were taken from the best preparations obtainable, regardless of the age.

(*b*) SPORES.—At first it was thought that *B. mesentericus* with small spores was quite sharply marked off from *B. vulgatus* with relatively much larger spores. In general, strains of *B. mesentericus* have much smaller spores than those of *B. vulgatus;* yet there are strains of *B. mesentericus* that have comparatively large spores and strains of *B. vulgatus* which have comparatively small spores. Spores probably do not vary as much as do the vegetative forms.

FLAGELLA

In staining for flagella the technic employed by Williams, as presented in Mallory and Wright (10),[1] gave the best results.

MOTILITY.—No trouble is experienced in observing motility in any of the strains studied. Besides the ordinary hanging drop, the scheme of cutting small blocks from cultures growing on thin agar in petri dishes, and inverting just as in a hanging drop over a concavity in a slide, is very instructive in studying form and movement. The germination of spores may also be studied by this method. The small blocks can easily be made to adhere to the surface of the cover slip by applying a small amount of sterile bouillon.

STAINING.—Basic carbol-fuchsin is quite satisfactory for staining and is used almost exclusively. In staining by Gram's method, young 24-hour-old agar cultures are used and the stain is prepared fresh each time as follows: 5 per cent carbolic-acid solution, 10 parts; saturated alcoholic solution of gentian-violet, 1 part; allow the stain to remain on the thin smear for 5 minutes; without rinsing put in a watch glass and cover with

[1] Williams' method for staining flagella:

1. Flood the cover glass with a mordant consisting of.
   Alumnol 1% solution 5 c c.
   Osmic acid 2% solution 5 c. c.
   Tannin 20% solution 5 c. c.
   Shake the mixture and add three drops of glacial acetic acid and shake again.
2. Apply the mordant less than one minute without heating Wash thoroughly in water.
3. Cover the preparation during about one minute with a 1% solution of $AgNO_3$ to which sufficient ammonium hydroxide has been added to keep the silver in solution.
4. Wash in water.
5. Wash with 0.6% solution of sodium chloride
6. Flood the preparation with a 30% solution of ammonium hydroxide and immediately wash in water.
7. Apply a few drops of Ortol photographic developer The directions for making up this developer come with the Ortol.
8. Wash in water.
9. Cover with a 1% solution of gold chlorid during a few seconds.
10. Wash in water and apply Ortol developer for a few seconds
11. Wash in water and cover with a 1% solution of mercuric chlorid for a few seconds.
12. Wash in water.
13. Apply Ortol developer for a few seconds
14. Wash in water and repeat the application of chlorid of gold, the washing and the application of the developer two or more times.

One can substitute other developers for the Ortol with apparently as good success. A trial of the method will serve to convince that it is not so long or complicated as a perusal of the directions would indicate.

Lugol's solution, of a rich port-wine color, for 2 minutes, then again, without rinsing, put in 95 per cent alcohol for 15 minutes to decolorize.

OXYGEN REQUIREMENTS.—In determining oxygen requirements, cultures are inoculated into a deep tube of 1 per cent glucose agar after thorough boiling to drive off oxygen. The tubes are cooled as rapidly as possible after inoculation. The presence or absence of growth at the surface and in the depth of the tube is noted.

ANIMAL INOCULATION.—Rabbits and guinea pigs were used for testing the pathogenesis of the organisms. Neither *B. vulgatus* nor *B. mesentericus* was found to be pathogenic for guinea pigs. *B. vulgatus* was not found to be pathogenic for rabbits. Inoculations were made subcutaneously.

THERMAL DEATH POINT.—One will be impressed by the great discrepancy in the results of the experiments of different investigators to determine the amount of heat required to kill organisms or their spores. Rosenau (12, p. 683), in a study of the temperature required to kill the tubercle bacillus, gives a table showing the results gained by 32 investigators, including his own. The results of only 8 of these investigators agree. Discrepancies equally as great occur in the results given for organisms other than the tubercle bacillus, as will be found on consideration of the literature. Such lack of agreement in results may be only apparent. It is not at all unlikely that the temperature at which organisms or their spores are killed varies under different conditions more than is generally supposed. Among the factors which may operate to cause such a variation in results may be mentioned the age of the culture, the kind of medium used, including the amount of moisture present, the length of time of incubation, the amount of culture heated, etc. If the results secured are to be of the greatest practical value, an attempt should be made to determine under what conditions organisms or their spores withstanding the greatest amount of heat are produced and exist. In the attempts to determine the thermal death point of *B. vulgatus* and *B. mesentericus* two methods were used. In one a suspension of spores was prepared in test tubes of bouillon loosely plugged with cotton (13). The tubes were placed in an open vessel of water of the temperature desired, and, after time was allowed for the tubes to acquire the temperature of the water bath, the heating was continued for the desired length of time at the desired temperature. The tubes were then removed from the water bath and cooled rapidly. In the other method, tubes of soft glass of about ⅛ inch internal diameter were drawn to a capillary tube at one end and in the middle, leaving two bulblike expansions. These were sealed in the flame at one end after drawing up the spore suspension. Owing to the fact that one capillary end was left open during the heating, this method can not be used for the boiling temperature, since some of the suspension is forced out, contaminating the water bath and consequently the outside of the other tubes. By

the use of these thin tubes, the spore suspension acquires the temperature of the water bath almost immediately. In testing *B. vulgatus* and *B. mesentericus* for the thermal death point, spores of varying age were used. In the earlier experiments, where the heatings were made for 10 minutes at temperatures of 90° C. and under, no record was made of the age of the spores. In these early experiments heating at 90° for 10 minutes did not kill the spores, as a growth was secured in the tubes of spore suspension after heating and on agar plates made from the tubes. In the later experiments, cultures of various strains of *B. vulgatus* and *B. mesentericus* on potato and agar slopes about 1 month old were used. In such cultures there are practically only spores, and it is likely that at this age they have lost little of their vitality. The greatest amount of heat applied was 100° C. for 20 minutes, with the result that growth occurred in some of the tubes containing spore suspensions of *B. vulgatus* and on agar plates made from the tubes. *B. mesentericus* seems not to survive the amount of heat that some of the strains of *B. vulgatus* do. Beyond these statements the writer does not wish to commit himself. It is believed that much careful work, with spores of known age from different strains on different culture media, and secured under different conditions of growth and development, will be necessary to establish the maximum amount of heat required to kill the spores of the organisms studied.

Bacillus mesentericus (Flugge), Lehmann and Neumann (8), Chester (2)
Syn. *Bacillus mesentericus fuscus* Flugge.

OCCURRENCE.—Found everywhere about the apiary, on combs, and on different parts of the hive. Found on culturing larvæ, both diseased and healthy.

MORPHOLOGY AND MICROMETRY.—Hanging-drop and hanging-agar-block cultures show a finely granular organism occurring in ones and twos and short chains of a dozen or so individuals. Rarely coarse metachromatic granules may be seen. The flagella are distributed over the body. The rods are smaller than those of *B. vulgatus*, measuring from 1.3 to 2.8 μ in length and from 0.4 to 0.8 μ in width. The average length, however, lies nearer the mean of the extreme lengths observed above. Spores are usually formed in the middle of the rod, but may be found nearer one end. They measure from 1.0 to 1.9 μ in length and from 0.5 to 0.8 μ in width.

MOTILITY.—The organism is actively motile.

GRAM'S STAIN.—It appears that the organism takes Gram's stain only partially.

OXYGEN REQUIREMENTS.—Aerobic and facultatively anaerobic, probably growing better at the surface of the deep glucose-agar tube than throughout the medium.

AGAR COLONIES.—The colonies at the surface are circular and usually smaller than those of *B. vulgatus*, although they may spread over the

plate, and are gray, raised, and glistening. Under a power of 90 diameters they are found to be finely granular, more dense at the center, where a distinct nucleus is seen. At the periphery the colonies fade out to a very delicate stippled appearance, the margin entire and clear-cut without the pronounced outgrowths as in *B. vulgatus*, although they may show long finger-like radiating lobes; they are also unlike *B. vulgatus* in lacking the characteristic convolutions and mottlings. (Pl. 93, *A*). Deep colonies in the gross are gray and pinpoint-like. Under a power of 90 diameters the colonies are dense, quite irregular in form, and occasionally show slight mosslike branching.

GELATIN COLONIES.—Colonies are at first barely visible. Under power these colonies are of all shapes, from circular to the most bizarre types imaginable, often resembling cockleburs with rather unusually long projecting spines. Surface colonies are circular in outline, and liquefy, with the formation of a gray membrane which floats on the surface of the liquefied area.

BOUILLON.—The growth forms a heavy turbidity with a luxuriant growth around the wall of the tube at the surface which often does not until later close over at the surface of the liquid to form a complete cup-like membrane of luxuriant growth usually much resembling ground glass. The membrane usually turns yellow later. The turbidity increases and the medium remains often heavily turbid for days; later the turbidity may clear from the sinking of the organism and this, together with the sinking of the ground-glasslike membrane, which may be re-formed several times after sinking, produces a large amount of viscid sediment which may be made to arise from the bottom in the form of a long twisted spiral on agitation. A luxuriant ring growth is usually left around the wall of the tube at the surface. The above characteristics of bouillon growth apply also to the various sugars—namely, glucose, lactose, saccharose, levulose, maltose, and the alcohol mannite. As regards the reaction in bouillon and the various sugars and mannite, it may be said that there is no sharp definite reaction, especially for mannite and plain bouillon. Usually an acid reaction may be found at the fifth and tenth days in glucose, levulose, maltose, and lactose, when carefully titrated with *N/20* sodium-hydroxid solution. Plain bouillon, mannite, and saccharose are less likely to show a perceptible change in reaction. So variable are these reactions that it would not be feasible to submit a type series.

GAS PRODUCTION.—No gas is produced in fermentation tubes of glucose or in deep tubes of glucose agar.

SERUM.—There is usually some liquefaction, the resulting liquid being light to dark amber in color.

POTATO.—The growth varies in the same strain at different times and in the different strains. Usually, however, the growth presents a characteristic reticulated or netlike appearance. Often the growth above is smooth, glistening, raised, and butter-like. The color of the growth is

yellow, brown, or a dull slate. To secure the characteristic netlike growth, there must be an abundance of water in the butt of the tube (Pl. 94, *B*).

AGAR SLOPE.—A gray, raised, glistening growth spreads rapidly over the surface of the medium. Later the growth turns brown or yellow, and a gray ground-glass cuplike membrane often forms at the butt over the water of condensation.

MILK.—A coagulum is formed usually after about the third day. Following this, there is partial digestion of the coagulum, which is never complete, as in *B. vulgatus*. The coagulum may soften or undergo change in consistency and rise in the tube later, so that a very thick, slimy liquid results.

AZOLITMIN AND LITMUS MILK.—Sometimes a change may be noticed in 24 hours, the color turning slightly pink; later changes are manifest either by the tube turning a deep wine-red color or forming a soft, curdy, pink coagulum. As in plain milk, the curd is not entirely digested, a residue always remaining at the lower part of the tube, or the whole tube is a brown or black, thick, slimy liquid.

GELATIN STAB.—Liquefaction is not evident usually until the second or third day; then a slow, stratiform liquefaction proceeds, usually complete after a month or six weeks. A gray membrane is formed at the surface; and in the liquefied medium there is a turbidity, and floating flakes are present. Later the medium clears, forming a large amount of sediment at the bottom of the tube.

FIVE PER CENT GLYCERIN GELATIN STAB.—Growth is sometimes very slight without liquefaction. Liquefaction, when it takes place, is much slower than in plain gelatin, and is incomplete, but there are the same growth characteristics as to membrane, turbidity, and sediment as in plain gelatin.

INDOL.—None observed.

NITRATE REDUCTION TO NITRITE.—Usually negative. Sometimes a positive reaction may be observed in a strain which usually gives a negative reaction.

Bacillus vulgatus (Flugge), Migula, Lehmann and Neumann (8)

Syn. *Bacillus mesentericus vulgatus* Flugge; *Bacillus A*. White (16). Common name, potato bacillus

OCCURRENCE.—Same as that of *Bacillus mesentericus*.

GELATIN COLONIES.—Colonies at first are barely visible to the naked eye. Under a magnification of 90 diameters deep colonies are found to be more or less circular, irregular, finely granular, and often quite dense, with mosslike outgrowths, or with long raylike processes. Surface colonies present at first a finely granular appearance, and later usually become folded and mottled; later a membrane, more or less cotton-like, floats on the liquefied area.

AGAR COLONIES.—If only a few colonies are on a plate, they spread rapidly to almost any size, are not circular, and do not manifest a clear-

cut margin, as do the colonies of *B. mesentericus*. The appearance is characteristic under a power of 90 diameters, a marked folding resembling the convolution of intestinal coils being present. Sometimes this folding is less pronounced and the colonies present a peculiar mottled appearance, showing distinct denser and lighter areas of growth (Pl. 93, *B*).

MORPHOLOGY AND MICROMETRY.—In the hanging-drop or hanging agar block the organisms are quite distinctly granular, usually large metachromatic granules showing distinctly. The organism occurs singly, in pairs, and in long chains. The flagella are distributed over the body. The rods are quite variable in size, being 1.3 to 5.0 μ long and from 0.6 to 1.3 μ wide. Spores are usually formed in the middle of the rod, although at times spores are found nearer one end. They measure from 1.1 to 2.5 μ in length and from 0.7 to 1.3 μ in width.

MOTILITY.—The organism is rather actively motile.

GRAM'S STAIN.—The organism is stained by Gram's method.

OXYGEN REQUIREMENTS.—Aerobic and facultatively anaerobic with best growth at or near surface of the deep Liborius tube.

BOUILLON.—Growth varies in bouillon. Usually sooner or later a luxuriant gray, ground-glasslike, wrinkled membrane forms, extending for a considerable distance up the walls of the tube. The medium becomes uniformly turbid, increasing in degree for a time and later clearing, usually at the time the membrane forms. Later from the sinking of the membrane and clearing of the medium a large amount of viscid sediment forms rising from the bottom of the tube as a spiral on agitation. The membrane at the surface may sink and be re-formed many times. As the membrane sinks, there is usually left at the surface of the tube on the walls a ring growth. The membrane is apt to turn yellow or brown toward the fifth day and onward. The above characteristics of bouillon growth apply also to the various sugar bouillons—namely, glucose, lactose, saccharose, levulose, maltose, and the alcohol mannite, not different essentially from *B. mesentericus*, except that the membrane is usually more luxuriant, more wrinkled, and turns brown or yellow sooner. The membrane is not, however, so characteristically ground-glasslike, and the medium clears sooner. As regards the reaction in bouillon and the various sugars and mannite, what was said under *B. mesentericus* applies to this organism.

GAS PRODUCTION.—No gas is produced in fermentation tubes of glucose or in deep tubes of glucose agar.

SERUM.—Some liquefaction was noted in some of the strains. A luxuriant, gray, glistening, raised growth appears over the whole surface within 24 hours. Later, the growth turns yellow or brown as liquefaction proceeds and the resulting liquid varies from light to dark amber.

POTATO.—A luxuriant, moist, gray, raised, much-wrinkled growth takes place, most pronounced in the butt, especially over the water.

Sometimes the growth is yellow, brown, or pink, imparting the same coloration to the potato. A liberal amount of water must be present in the tube to secure the luxuriantly wrinkled and folded characteristic growth (Pl. 94, *A*).

AGAR SLOPE.—A luxuriant, gray, raised, glistening, moist growth spreads over the whole surface. Often the growth is more or less wrinkled. Later the coloration is brown or yellow, with occasionally a slight coloration of the medium near the surface.

MILK.—Most strains exhibit a firm coagulum within 24 hours, then the coagulum is gradually digested, the band of turbid whey gradually extending toward the bottom until the whole tube is a thick liquid. Often there are floating flakes or masses, and a viscid sediment.

AZOLITMIN AND LITMUS MILK.—Usually a pink coagulum is formed within 24 hours; later this is digested so that a gradually extending zone of turbid wine-red liquid is left above; still later the whole tube becomes a thick brown liquid with floating flakes or masses and a viscid sediment. At times no firm coagulum is noted; but the first change observed is the deep wine-red turbid liquid, which later turns brown. The changes in litmus milk show greater variation than in plain milk. The turbid brown coloration may appear early without the previous turbid wine-red coloration, or the brown liquid may be streaked with red or violet, or the tube may be a turbid brown liquid at first and later assume the red or violet coloration as a whole or in streaks.

GELATIN STAB.—Growth is usually manifest within 24 hours. Liquefaction proceeds rather rapidly, and is infundibuliform, as a rule, but usually becomes stratiform later. The medium is turbid, with a luxuriant membrane at the surface and growth extending up the side of the tube. Later the membrane sinks and the liquefied medium clears, forming a large amount of sediment, which is flaky or cotton-like in appearance.

FIVE PER CENT GLYCERIN GELATIN STAB.—Same as plain gelatin, except that the growth is slower and usually less luxuriant.

INDOL.—None observed in any of the strains.

NITRATE.—Reduction to nitrite is positive, with a strong red coloration on adding Greiss's reagent.

DESCRIPTION OF A STRAIN FROM SAMPLE 2329

In general, the cultural features and the morphology of the strain from sample 2329 resemble those of *B. vulgatus*, especially in the wrinkled and coiled growth on potato. On agar plates, however, this strain has never manifested the characteristic folding or mottling of *B. vulgatus* as seen under a power of 90 diameters, always presenting a nearly even homogeneous structure, with a strikingly clear-cut periphery, and usually as finely granular structure as in *B. mesentericus*. Moreover, there is not the same tendency to a spreading growth, the colonies usually being small and decidedly convex or pulvinate, and often on agar presenting a

decidedly yellow coloration. On gelatin the colonies are always of a pronounced yellow color. In the slowness with which it liquefies gelatin and coagulates milk and in its not reducing nitrates to nitrites, it resembles *B. mesentericus*. It is a large granular organism with metachromatic granules and has large spores, thus suggesting *B. vulgatus*.

Bacillus orpheus White

OCCURRENCE.—Encountered as an occasional secondary invader in larvæ infected with European foulbrood.

AGAR COLONIES.—Surface colonies small, often barely visible. Under a magnification of 90 diameters the colonies show a delicate, finely granular structure. Surface colonies may spread to any size, and a spreading growth may even cover the whole agar plate. Such growth is usually rather delicate and of a pale bluish tinge. Deep colonies are denser, irregular in form.

MORPHOLOGY.—In the hanging-drop the organism has a granular appearance, and is more slender than *B. vulgatus*. It occurs singly, in pairs, and in long chains, the division line separating individual organisms being difficult to make out. The ends of individual organisms are rounded markedly, appearing almost bluntly pointed. The spores are excentrically placed, and measure from 1.1 to 2.2 μ in length and from 0.6 to 1.2 μ in width.

MOTILITY.—The organism is rather actively motile.

GRAM'S STAIN.—The organism does not stain by Gram's method.

OXYGEN REQUIREMENTS.—Aerobic and facultatively anaerobic.

BOUILLON.—Frequently no growth of importance takes place until after 24 hours. The medium becomes turbid; often a luxuriant, gray, wrinkled membrane forms, and there are usually floating flakes. The medium is likely to clear later, with the formation of a considerable amount of sediment. The above characteristics of bouillon growth apply also to the various carbohydrate media—namely, glucose, lactose, saccharose, levulose, maltose, and mannite. On titration there are no definite reaction changes in the carbohydrate media.

GAS PRODUCTION.—No gas is produced in fermentation tubes of glucose bouillon or in deep tubes of glucose agar.

SERUM.—A luxuriant, raised, reticulated growth occurs. There is little, if any, liquefaction, and the water in the butt supports a gray membrane.

POTATO.—The growth on potato is slight and practically invisible.

AGAR SLOPE.—Within 24 hours separate areas of a delicate growth occur. Later the areas become confluent, and a luxuriant, gray, raised glistening growth appears over the whole surface.

MILK.—Milk is coagulated after several days. The coagulum is then slowly digested.

AZOLITMIN MILK.—The color is soon discharged to a dull pink or brown, coagulation taking place later. The coagulum is slowly digested.

GELATIN STAB.—Liquefaction proceeds slowly until complete. A gray membrane forms at the surface, and gray flakes float in the liquefied medium.

INDOL.—A very strong pink coloration takes place on adding the reagents.

NITRATE.—Reduction to nitrite was positive for one of the two strains tested and negative for the other.[1]

The description of *B. orpheus* is not as complete as it might be made. This is owing to the fact that it was only decided rather late in the course of preparation of the paper to include a description of *B. orpheus* at all.

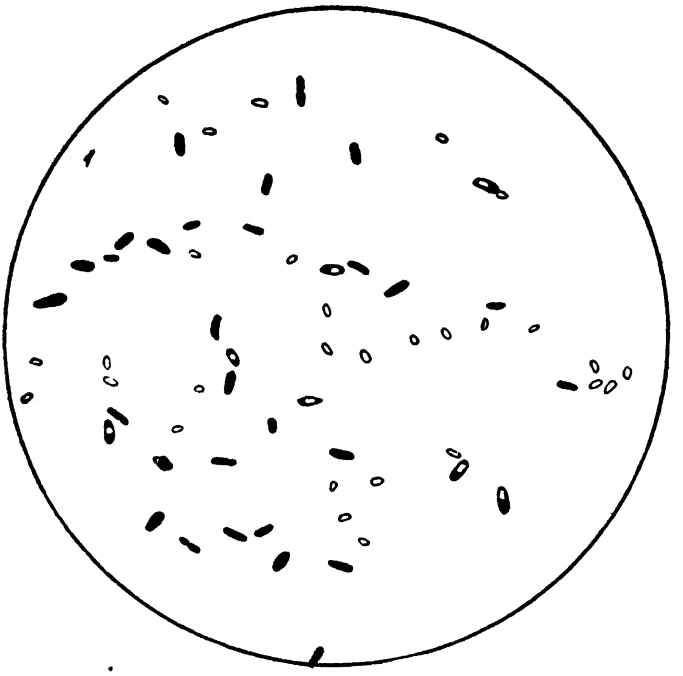

FIG. 1.—*Bacillus mesentericus:* Smear from an 18-hour-old agar slope [2] × 1,480.

The description is based on the behavior of two different strains in the different media. White (18) discovered and named this organism in 1912.

The following brief characteristics should aid in the identification of these spore-forming organisms:

(*a*) *B. vulgatus* forms a heavy, wrinkled growth on potato (Pl. 94, *A*); *B. mesentericus*, a less luxuriant, netlike growth (Pl. 94, *B*).

(*b*) Even under low magnification, cultures on agar plates show that *B. vulgatus* (Pl. 93, *B*) grows in curves much resembling *B. anthrax*, while

[1] In old cultures of agar and plain milk a decided green coloration of the medium is noted.

[2] The drawings reproduced in figures 1 to 6 and Plate 93 were made with the aid of a camera lucida, the image being projected to the base of the microscope stand. The photograph of the potato cultures was made for the author by Dr. James A. Nelson, of the Bureau of Entomology.

colonies of *B. mesentericus* (Pl. 93, A) present a finely granular or stippled appearance.

(*c*) *B. alvei* and *B. orpheus* both grow slowly on potato; often it is almost impossible to detect any growth at all by the naked eye (Pl. 94, *C*, *D*), but stained smears must be made from the slope (fig. 3).

(*d*) The microscopic appearance of stained smears in the spore stage should be an important differential aid. Spores of *B. alvei* almost invariably show a vestige of the rod remaining (fig. 4); this does not occur in *B. vulgatus* (fig. 2), *B. mesentericus* (fig. 1), or *B. larvae* (fig. 5). The spores of *B. orpheus* (fig. 3) are distinctive, being excentrically placed.

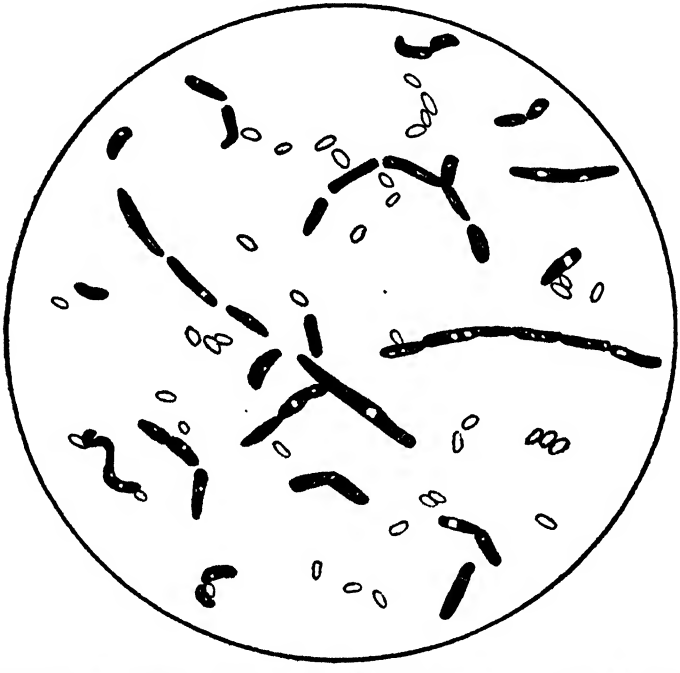

FIG. 2.—*Bacillus vulgatus:* Smear from a 9-day-old agar culture × 1,480. The rods are morer regular in younger cultures.

(*e*) *B. larvae* does not grow on the ordinary media of the laboratory, but requires media prepared from the juices of bee larvæ.

(*f*) Inasmuch as *B. pluton* occurs constantly and in such large numbers in European foulbrood larvæ, it was thought well to include a drawing of the organism (fig. 6).

PART II.—INOCULATION EXPERIMENTS

In the inoculation experiments not only the definitely different and typical species of *B. vulgatus* and *B. mesentericus* were used but also those atypical strains not partaking so strictly of specific biological characteristics. As will be seen from Table I, the inoculations include

the organism isolated from sample 2329, the strain of *B. vulgatus* secured from the Bureau of Animal Industry, and the strain of *B. vulgatus* isolated from potato.

MEDIA USED FOR GROWING THE CULTURES

Cultures were grown upon agar slopes prepared from both bee-larvæ bouillon and plain peptonized beef bouillon. The ordinary agar was prepared as usual by adding 1.25 to 1.50 per cent of shredded agar to beef bouillon titrated to +1.5 per cent to phenolphthalein. The bee-larvæ

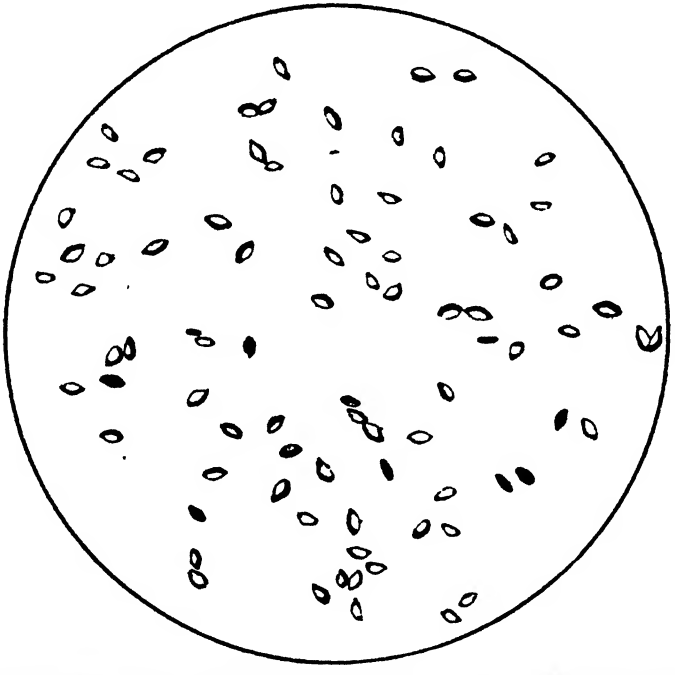

FIG. 3.—*Bacillus orpheus:* Smear from a 4-day-old agar culture × 1,480. Note the excentrically placed spores

agar was prepared by picking young bee larvæ from the combs, crushing, straining through cheesecloth, adding three times the volume of water, and then, as in the case of beef bouillon, heating in a water bath at a temperature of 60° to 65° C. for one hour. After filtering, peptone and sodium chlorid are added, and the reaction adjusted. To this, bee-larvæ bouillon titrated to +1.5 per cent to phenolphthalein, 1.25 to 1.50 per cent of agar is added.

AGE OF CULTURES AND METHOD OF INOCULATING

The age of the cultures at inoculation varies considerably. The usual method of securing cultures for inoculation is to sow the whole number of slopes designed for one colony at one time. Usually 30 slopes are fed

to a colony. These are given at the rate of 3 a day for 10 days. By this procedure the cultures at the last, or tenth inoculation, are 10 days older than those at the first inoculation, assuming that the three slopes were always given on every consecutive day without a break. The age of cultures at the first inoculation varies considerably, ranging from 1 day to 16 days. Sometimes the interval between inoculations for one or more times is greater than a day, thus advancing the age of the culture at the last inoculation considerably. Only one colony was inoculated with a fresh 24-hour-old culture at each feeding, since it was found that most cultures do not produce spores in abundance until somewhat older.

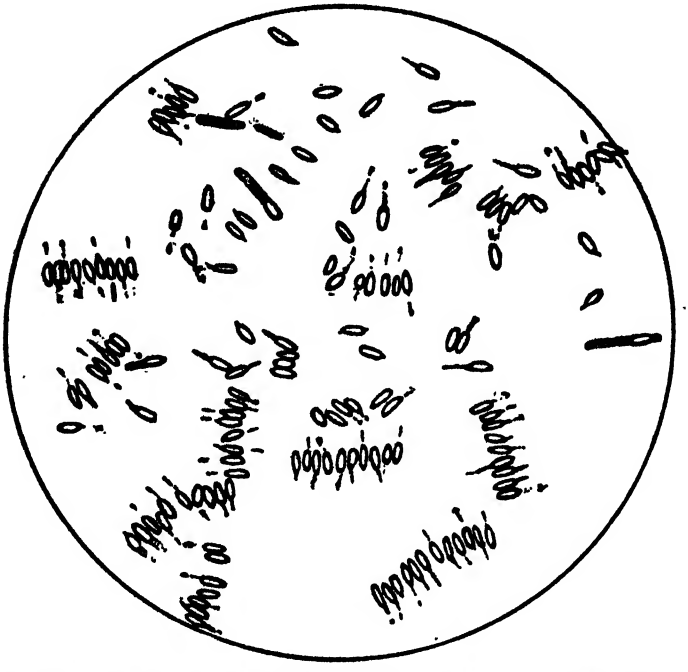

FIG. 4.—*Bacillus alvei:* Smear from a 48-hour-old agar culture × 1,480. Note the vestige of rod clinging to nearly all of the spores. Note also the tendency of the spores to occur in rows.

While 3 slopes for 10 days was the usual allotment of a colony under inoculation, this was varied at times so that some colonies received a somewhat less number than 30 agar slopes of culture.

The cultures were inoculated by feeding to the bees in sterile sirup, washing the culture off of the slopes with sterile physiological salt solution by the use of a sterile glass rod. The cultures were fed when the organisms were forming spores in abundance.

DISCUSSION OF INOCULATIONS

Table I gives various data as to the number of colonies inoculated, kinds of media used, number of different strains, number of different

hives, seasonal range, and various other data by separate years, together with totals of same for the whole five years' inoculations.

TABLE I.—*Results of inoculation experiments with organisms of the Bacillus vulgatus group* [a]

Year.	Number of different strains.	Number of equivalent colonies fed cultures on—		Total inoculations equivalent to colonies.	Number of strains repeated—i. e., used on both bee-larvæ and ordinary agar.	Number of different colonies.	Seasonal inoculation range	Number of slopes of agar.		
		Bee-larvæ agar.	Ordinary agar.					Bee-larvæ agar	Plain	Total.
1911.....	6 (2329, 2094, 2291, 2442, *B. vulgatus* B. A. I., bacillus from potato).	6	[b] 4	10	[b] 4	4	July 26–Sept. 16.	168	117	285
1912.....	4 (2904, 2905, 3309, and 3335).	[c] 2	4	6	[c] 2	6	June 28–Sept. 25.	60	118	178
1913.....	6 (3932, 3969, 4102, 4107, 4019, and 4016)	0	6	6	(*d*)	[e] 2	June 30–Sept. 27.	.....	174	174
1914.....	8 (4277, 4288, 4292, 4323, 4381, 4429, 4438, and 5356).	0	8	8	(*d*)	*f* 4	June 12–Oct. 5...	....	218	218
1915.....	4 (4473, 4709, 4727, and 4823).	4	0	4	(*g*)	2	Aug 2–Sept. 21.	107	...	107
Total.	28....	12	22	34	6	18	June 12–Oct. 5.	335	627	962

[a] Culture numbers refer to the laboratory numbers of samples of brood from which culture was obtained.
[b] 2329, 2094, 2291, and 2442
[c] 2904 and 2905.
[d] Used only on ordinary agar.
[e] Three used also last year
[f] One of these fed three cultures: 4288, 4323, and 4438
[g] Used only on bee-larvæ agar.

In a consideration of the number of colonies inoculated it will be seen that a different colony was not always used for each strain. This was not necessary or even desirable, since, if one strain shows no pathogenic effects upon feeding to a colony, the same colony may be used over and over again, as often as convenient, for the inoculation of other strains. There were used, for the five years, 18 different colonies, which, with the repeated inoculations of some colonies, brings the equivalent number of colonies up to 34. Twenty-eight different strains of organisms representative of the *B. vulgatus* group were inoculated. Twelve of these cultures were grown on bee-larvæ agar and 16 on ordinary agar, as prepared from beef bouillon. Six of the 28 strains were grown upon both ordinary agar and bee-larvæ agar. The total number of slopes inoculated was 962, of which 335 were on bee-larvæ agar and 627 on ordinary agar. The seasonal range of the inoculation extends from June 12 to October 5, which is the seasonal range of the inoculations for the year 1914, as can be seen from Table I.

RECOVERY OF THE ORGANISMS INOCULATED

In order to determine the presence of the organisms in the inoculated colonies, cultures of the young brood were made at frequent intervals. This was done by gently removing the larvæ from the cells and first washing in five or six changes of sterile water for the purpose of removing the organisms present in the food surrounding the grubs as they lie in the cells. After this the larvæ were crushed on a sterile microscope slide, and a loop of the crushed contents was plated out on an agar plate. By the estimation of the number of organisms on the agar plate after 24 hours'

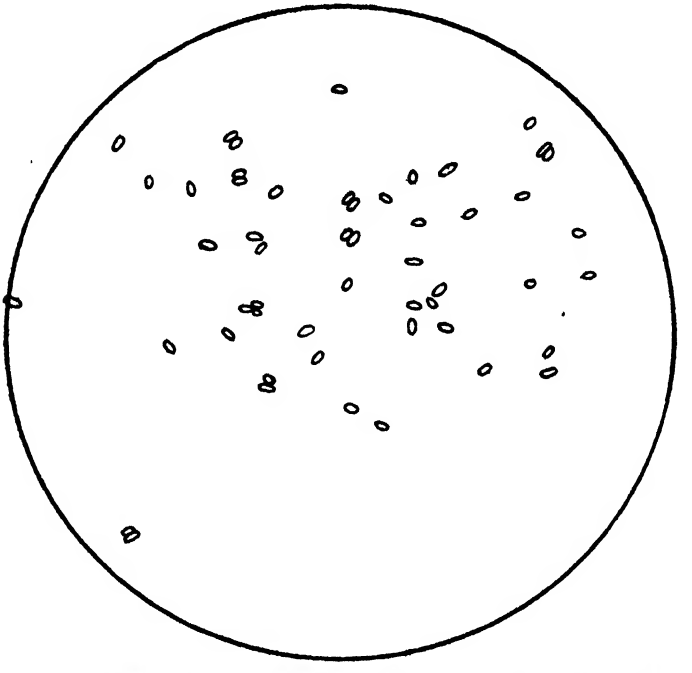

FIG 5.—*Bacillus larvae:* Smear direct from a diseased larva. × 1,480. Note the small size of the spores as compared with those of *B. alvei*, the lack of any vestige of rod, and the lack of any tendency of the spores to occur in rows.

incubation, a fair idea is obtained of the number of organisms ingested. Of the 34 equivalent colonies inoculated, the organisms were in every instance recovered in abundance by culturing the larvæ. A loop of the crushed larvæ, when inoculated onto agar plates, often yielded colonies too numerous to count. The organisms were recovered in large numbers as early as the next day after the first inoculation. No exhaustive study was made to determine the length of time after the last inoculation that the organisms could be recovered, though in several instances they were cultured from the larvæ as late as eight months after; but the organisms were not in sufficient numbers to warrant the conclusion that their presence was due to the previous experimental inoculations. Nor-

mal larvæ were found to be nearly always sterile. This fact was pointed out by White in 1906 (16, p. 16, 29).

The writer has found occasionally a few organisms, both of *B. vulgatus* and *B. mesentericus*, on culturing apparently healthy larvæ, and on still rarer occasions a very large number in a single larva. This should excite no surprise when the great abundance of both of these organisms in nature, especially in water, is considered. Another method of determining the presence of the bacteria in the larvæ is by the study of paraffin sections. Larvæ were not sectioned from every colony inoculated, but enough sections were secured to show that the organisms were present in great

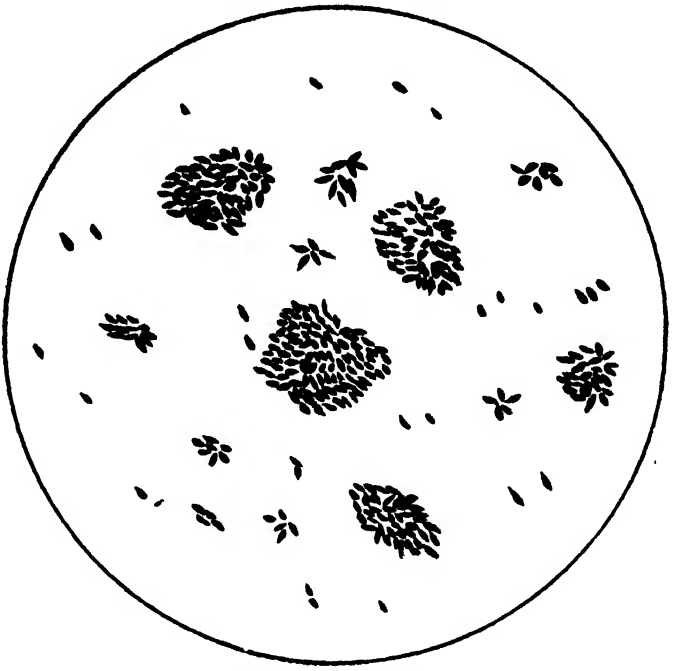

FIG. 6.—*Bacillus pluton:* Smear direct from a diseased larva ×1,480 Note that the organism stains uniformly, this distinguishing it from any spore-forming bacterium of the apiary

abundance in the alimentary canal of the larvæ. It was hoped by sectioning larvæ and pupæ, as well as the shed skins and detritus in the bottom of the cell, that the ultimate fate of the bacteria in the gut of the bee might be learned. The results of such investigations are not definite enough for publication at this time. Indications point to the elimination of the bacteria some time after the brood passes to the pupal stage, since so comparatively few organisms were ever recovered from culturing the contents of pupæ, especially later pupal stages.

RESULTS OF THE INOCULATIONS

In none of the colonies inoculated over the whole five seasons of feeding of the cultures was there any appearance of the brood simulating either

American foulbrood or European foulbrood; nor were any other abnormal conditions detected. If we judge from the amount of culture or virus required to produce disease generally, more than enough of the culture was fed to produce disease. Also, the seasonal range is certainly sufficient to cover the time of appearance of any of the known infectious brood diseases of bees as occurring naturally, or by inoculation with the proper etiological factor. All of the known infectious brood diseases of bees—American foulbrood (17), European foulbrood (18), and sacbrood (19)—have been thus produced experimentally. Unfortunately Lambotte (7) does not describe sufficiently the method of preparing his agar medium from the juices of bee larvæ, and a medium might be devised to produce the variation claimed—that is, transformation of *B. mesentericus vulgatus* to *B. alvei*. But, if it is granted that Lambotte fed cultures of *B. alvei*, his results would not agree with present-day investigations, since *B. alvei* has been found not to produce disease upon repeated inoculation. Mindful of the variation possible in different strains of the same organism from a study of the *B. vulgatus* group, it seems, nevertheless, that the differences in biological characteristics of *B. alvei*, *B. vulgatus*, and *B. mesentericus* are too great to warrant expecting any transformation of one to the other in any short period of time.

SUMMARY

(1) Lambotte (7), in 1902, claimed to have produced "foulbrood" of bees by feeding them cultures of *B. mesentericus vulgatus*.

(2) Lambotte, in explanation of the above, states that *B. alvei* arises as a special variety of *B. mesentericus vulgatus* when the cultures are grown upon media prepared from the juices of bee larvæ.

(3) There are at least five spore-forming bacteria occurring commonly about the apiary—*B. vulgatus*, *B. mesentericus*, *B. orpheus*, *B. alvei*, and *B. larvae*.

(4) Mistakes in the identification of the above-mentioned organisms might well be made, especially without knowledge of their occurrence.

(5) There seems no doubt that many of the earlier investigators of bee diseases confused the identity of the spore-forming bacteria of the apiary.

(6) From our present knowledge of these organisms, their identification should be a matter of less difficulty.

(7) The author, from his study of *B. vulgatus*, *B. mesentericus*, and *B. alvei*, believes that the biological differences of the three species are too great to warrant expecting the transformation of one to another in any short period of time.

(8) Even if it is granted that Lambotte fed pure cultures of *B. alvei*, his results would not agree with those of present-day investigators of bee diseases, since *B. alvei* has not been found to produce disease in bees upon repeated inoculations.

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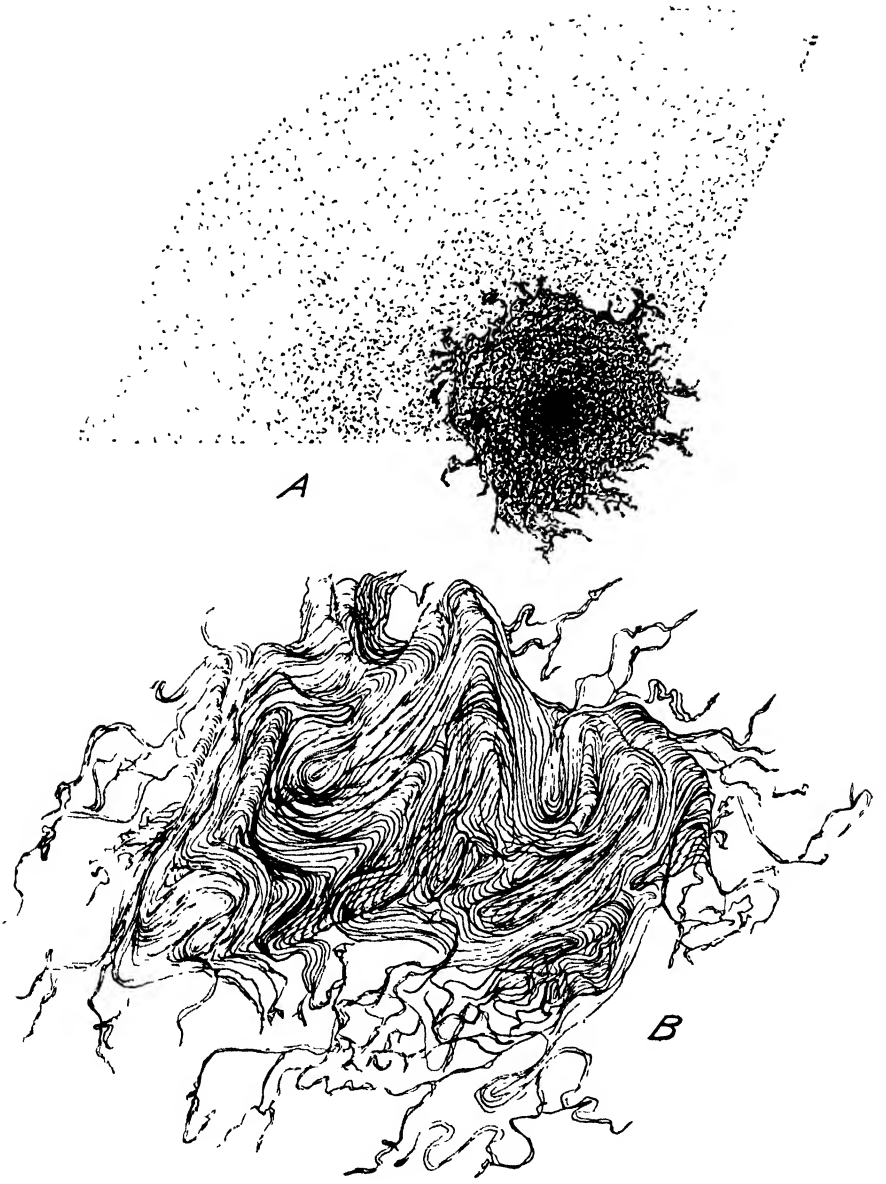
PLATE 93

A.—*Bacillus mesentericus:* Growth of a 24-hour-old surface colony on an agar plate. × 140.

B.—*Bacillus vulgatus:* Growth of a 24-hour-old surface colony on an agar plate. × 140.


A
B

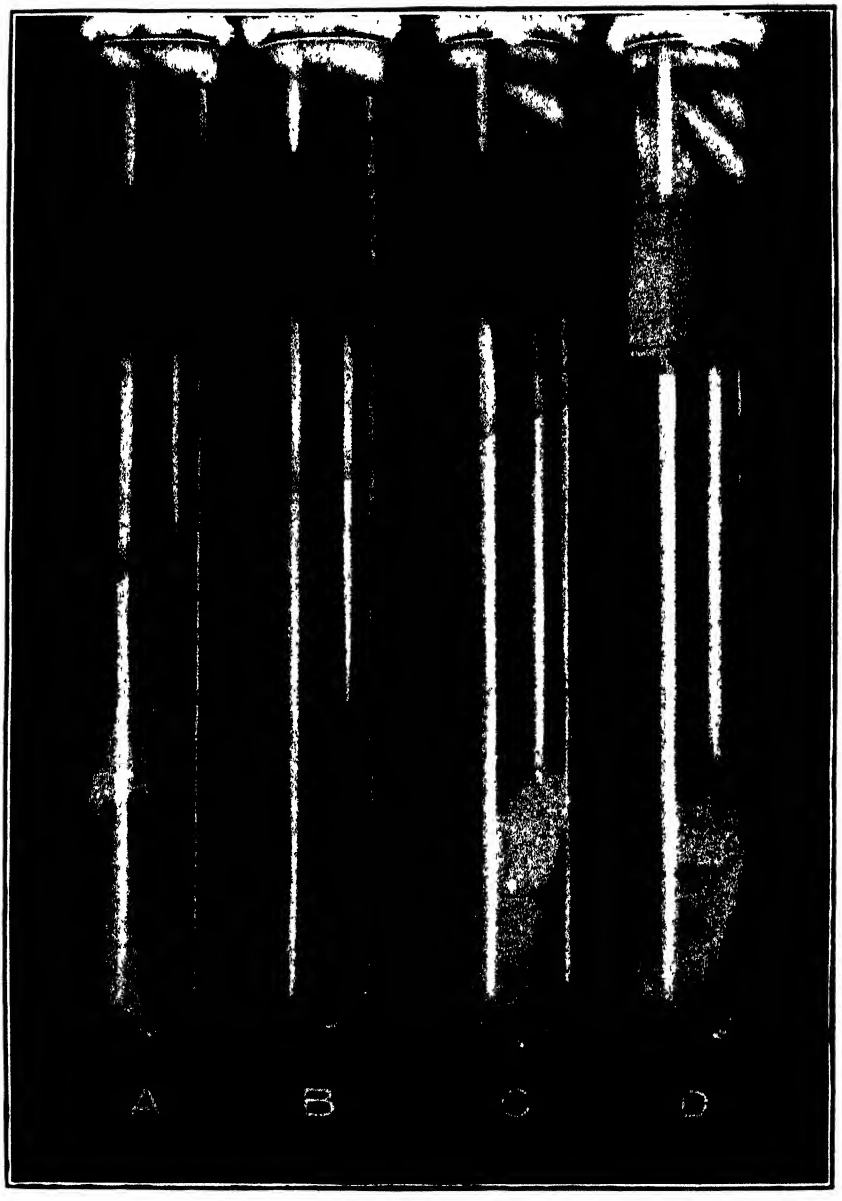

A
B
C
D

PLATE 94

Growth of 24-hour-old cultures on potato slopes. All tubes contain a liberal quantity of water in the butt.

A.—*Bacillus vulgatus:* Note the luxuriant folded and wrinkled growth, especially on the surface of the water in the butt, and on the lower portion of the slope.

B.—*Bacillus mesentericus:* Note the less luxuriant growth. The growth on the surface of the water is more in the nature of a thin pellicle without any folding or wrinkling. Often a netlike appearance of the growth is noted.

C.—*Bacillus alvei:* No visible growth.

D.—*Bacillus orpheus:* No visible growth.

FUSARIUM-BLIGHT, OR WILT DISEASE, OF THE SOYBEAN[1]

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INTRODUCTION

During the summer of 1915 a package of diseased plants of the soybean (*Soja max* (L.) Piper (17);[3] syn. *Glycine soja*, *Soja hispida*, etc.)[4] was received from a correspondent at Red Springs, N. C. A large number of plants in the field from which these specimens were taken had become chlorotic, or were dead. The plants received were still green and in good condition for examination. The evidence obtained from a preliminary inspection indicated that the diseased condition was due to the presence of a fungus belonging to the genus Fusarium. Furthermore, nearly all of the isolations from this material gave pure cultures of a species of Fusarium.

The studies herein reported were therefore undertaken (1) to determine the parasitism of this species of Fusarium on soybean, (2) to establish its relationship to the Fusaria of the section Elegans in so far as a comparison of the cultural characteristics permit, and (3) by means of cross inoculations and field studies to determine the relationship of this disease of soybeans to the wilt disease of cowpeas (*Vigna sinensis* Hassk.) caused by *Fusarium tracheiphilum* Smith.

ECONOMIC IMPORTANCE OF THE SOYBEAN

The soybean is a native of tropical Africa, Asia, and Australia (23, p. 360–361; 17, p. 76) and was introduced into Europe by Kämpfer about 1690 (18, p. 9). At the present time it is the most important legume grown in Japan, China, and Manchuria. Its culture in England was begun in 1790. The plant was introduced into the United States from Japan in 1860. Since that time its cultivation as a soil-improving and a forage crop has been confined for the most part to the Southern States. North Carolina is probably foremost among these States in the production of soybeans. The yield in 1909 was 13,313 bushels,[5] and in 1915 was estimated[6] as approximately

[1] Published with the permission of the Director of the North Carolina Agricultural Experiment Station.

[2] The writer wishes to express his deep appreciation to Prof. H. R. Fulton, under whose direction the study was begun, and to Dr. F. A. Wolf, for his helpful suggestions and criticisms during the major part of the investigations and for aid in the preparation of the manuscript.

[3] Reference is made by number to "Literature cited," p. 438–439.

[4] For a complete synonymy, see Piper (17).

[5] U. S. Bur. Census, 13th Census, 1910, 1913. Statistics for North Carolina, p. 632.

[6] Estimate furnished by the North Carolina Experiment Station.

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1,000,000 bushels. Within the last two or three years this crop has become increasingly important because of the variety of products manufactured from the oil and meal.[1] During 1915, $9,000,000 worth of oil alone was imported. Local cottonseed-oil mill owners have been induced, however, partially by the efforts of the North Carolina Experiment Station, to crush soybeans during their otherwise idle season. The few mills in the State which have done this have found a ready market for the oil and meal.

OTHER SOYBEAN DISEASES

Soybeans are very generally observed to be quite free from disease, and no very seriously destructive parasites of this host appear to have been reported in the literature at hand. Of those reported, a detailed study has not been made, except in the case of *Bacillus lathyri* Manns and Taubenhaus (13, 14). The accounts of the others consist of brief fragmentary mycological notes and mention of their place of collection or of their appearance. Since any of them may appear on plants affected with blight or wilt, it is deemed advisable to call attention to the published accounts of these diseases and the appropriate bibliography.

Septoria sojina v. Thümen (on living or declining leaves) (24).
Phyllosticta sojaecola Massalongo (15, p. 688).
Aecidium glycines P. Henn. (6, p. 52).
Uromyces sojae (P. Henn.) Sydow (22, p. 429).
Bacillus sp. (on leaves)—Heald (9, 10), Smith (21), and Clinton (4).
Bacillus lathyri Manns and Taubenhaus (on leaves and pods) (13, 14), and Manns.
Heterodera radiciola—Scofield (19, p. 9), Gilbert (8, p. 9), Bessey and Byars (2, p. 8). (These authors merely mention the soybean as a host for this parasite.)
Chlorosis and crinkling (cause?) (Description of the disease in the field)—Clinton (5).
Septoria glycines T. Hemmi (comparison with *S. sojina* above) (11).

It is not believed that the presence of any of these organisms would lead to confusion in the diagnosis of blight caused by the species of Fusarium under consideration.

HISTORY AND OCCURRENCE OF THE DISEASE

No published report of a disease of soybeans caused by any species of Fusarium and one account only of attempts to produce a disease of this host with the cowpea wilt organism have been brought to the writer's attention. Orton (16, p. 16–19) conducted these tests at Edisto Island, S. C., in 1900, and at Monetta, S. C., in 1901. Several varieties of cowpeas and soybeans were planted on soil badly infected with the

[1] The following is a list of the most important products obtained from soybeans or in which soybeans enter: Soybean milk, meal or flour, soups, pork and beans, meat substitutes, fertilizer and cattle feed from the meal, and dynamite and high explosives, soaps, linoleum, rubber substitutes, margarine, Japanese sauce, paints, varnishes, toilet powder, waterproof cloth, salad oil, lubricants, and lard substitutes from the oil.

cowpea-wilt organism and with a nematode (*Heterodera radicicola*). Concerning the work at Monetta, S. C., he says (p. 18):

> Eight varieties [soybeans] were tried on ten plats. All proved to be immune to the wilt disease, but none of them was adapted to the local conditions. The growth was very small, the plants averaging from 8 to 14 inches high, though most of the varieties bore a good crop of seed for such small plants. All suffered from much drought in midsummer and all were badly injured by the root nematode. On examination of the roots a moderate number of bacterial tubercles were found. * * * They [soybeans] were at a considerable disadvantage in this test on account of the late date of planting and the ensuing dry weather.

The varieties tested were Tokio, Buckshot, Yosho, Ito San, Manhattan, Guelph, and Amherst. Orton reported that at Edisto Island the soybean made a heavy growth, 3 or 4 feet high and was free from the wilt disease. It may be said that a very considerable proportion of the several varieties of cowpeas grown in adjacent plots succumbed to wilt. The results of these tests accord with the observations of others who have had opportunity to observe these crops when they were grown on soil known to be infected with cowpea wilt.

A limited number of careful observations have therefore been made during 1915 and 1916 to determine whether the wilts of these two hosts are coextensive in range and thus to furnish evidence of the identity of the two. Two 5-acre fields on widely separated parts of the North Carolina Experiment Station farm, in which cowpeas and soybeans were grown in alternate rows, showed a very considerable proportion of the former host affected, whereas the latter remained entirely free from disease. In other localities of the State, soybeans growing on soil infected with the cowpea-wilt organism have remained disease-free.

Observations differing from these were made in the case of soil brought from another part of the Station farm. When this soil was used to grow soybeans in pots out of doors, it was found to be infected with soybean-blight, as shown by the development of the disease in 33 of the 80 pots. Wilt, both of cowpeas and soybeans, was present on the farm of the correspondent previously referred to, at Red Springs, N. C. Many of the soybean plants in this field were killed and many only stunted, so that a decrease in yield of 60 per cent during the past season is probably a correct approximation of his loss. Blight or wilt of soybeans has also been found to occur at Exum and Belhaven, N. C., and was the cause of considerable loss in both locations.

Since cowpea-wilt has been found in many localities throughout the Piedmont and the Coastal Plain sections of North Carolina, it is entirely probable, if we judge from the results to be presented subsequently, that the soybean-blight may appear more or less generally wherever the soil is infected with *Fusarium tracheiphilum*. Records received from the Office of Plant Disease Survey show that, up to the close of 1915, *F. tracheiphilum*

philum has been reported as being productive of losses to cowpeas ranging from 2 to 100 per cent in Indiana, Missouri, Mississippi, Louisiana, Texas, Oklahoma, Georgia, Florida, North Carolina, South Carolina, and Virginia.

APPEARANCE OF THE BLIGHT IN THE FIELD

In 1916, soybeans were planted during the last two weeks in May. This is somewhat later than usual, being due to the late season and a period of drought. When the plants were 4 weeks old, they had attained a height of 2 to 3 dm. and were apparently still free from disease. The disease was first observed on July 25, when the affected plants were about 8 weeks old. Symptoms of the trouble could probably have been found a week or two earlier. Affected plants, all of the same age but varying in height from 2 dm. to 1 meter, were observed on the 25th. The fungus is believed to have stunted these small plants. In no case has the disease been observed on seedlings.

The contrast in appearance of five healthy and five diseased plants is shown in Plate 95, *D*, *E*. The same type of clay soil was used in both pots, and the plants in each were grown out of doors under the same conditions. The plants shown in figure *E* were naturally infected from the soil. A considerable number of the leaves have fallen from the diseased plants, a portion of the petioles persist, the plants are dwarfed, and there is no evidence of wilting in any part of the plants. The foliage which persists on these plants is yellow as contrasted with the normal leaf green of healthy plants.

The occasional absence of a definite wilting of the leaves has been noted in other wilt diseases. Orton (16, p. 10), in speaking of the cowpea disease caused by *F. tracheiphilum*, says:

> The term "wilt" is somewhat misleading, as the leaves usually drop off before there is any conspicuous wilting. The name was applied because of its relationship to the wilt of cotton and watermelons, where this symptom is very prominent, and it seemed desirable to retain it for the cowpea disease.

In the case of the soybean disease, wilting is a less prominent symptom than in cowpeas, and is very seldom present at any stage of the disease. The plants, as a rule, drop all of their leaves and die without any evidence of wilting. Wilting has been observed in a very few instances in the field in the case of young plants. The woody nature of the stem and petioles probably accounts for the general absence of wilting in them, and the presence of well-developed mechanical tissues in the leaflets may account for their failure to manifest wilt. The possibility exists, also, that the physiological interaction of parasite and host differs from that exhibited by wilted cotton and watermelons infected with *Fusarium* spp.

Instead of applying the name "wilt," therefore, to the soybean trouble, it is perhaps desirable to call it "blight or wilt," the former

word describing the most prominent symptom on the foliage and the latter retaining the idea of its relationship to other wilt diseases produced by species of Fusarium.

Soybean blight or wilt may make its appearance on individual plants, but does not cause the death of all the plants within definite areas, as in the case of cotton wilt.

Although no definite effort has been made to determine the method of entrance of the organism into the host, it is thought that it enters through the smaller roots in practically the same manner as that described for other diseases of this character. Many of the fibrous roots are destroyed, and new roots are formed of insufficient number, however, to maintain the life of the plant.

Perhaps the most prominent symptom is a browning or blackening of the interior of the stems and roots. As soon as the leaflets begin to drop, this discoloration is evident when the root and stem are split longitudinally. This character is shown in Plate 95, figures *B* and *C*, showing healthy and diseased stems, respectively. As the disease progresses, the discoloration extends upward in the stem for one-half or more of its length. The tracheal tubes of affected stems when cut obliquely show as brown spots. The relative amount of discoloration in general and the depth of color in affected xylem portions is less in soybeans than in cowpeas.

A large number of stained free-hand sections were made of stems at all stages of development of the disease. In the early stage, only the xylem tubes nearest the pith were found to contain the fungus filaments. The pith had disappeared in both normal and diseased plants of moderate size. Later, other of these tubes throughout the xylem area are penetrated and become filled to a large extent with a network of fungus filaments. In still more advanced stages, all of the xylem elements (fig. 1, *A–G*) were found to contain the fungus; and, in addition, the cortical parenchyma was invaded.

The surface of stems of plants in advanced stages of the disease generally have salmon-colored spore masses, sporodochia, thickly and irregularly distributed over them.[1] This character is shown by the roughened appearance of the stem in Plate 95, *A*. The spore masses are composed of macroconidia of the fungus and are frequently found to occur on plants whose upper leaves are still healthy in appearance. Sometimes they are formed only in more advanced stages of the disease.

MANNER OF INFECTION AND SPREAD OF THE DISEASE

As stated above, the fungus may enter the plant through the small roots. In addition to the spread of the organism through the soil, spores are so abundantly produced that drainage water, implements,

[1] Sporodochia on stems of cowpeas are reported by Orton (16, p. 9) to appear after the death of the plants.

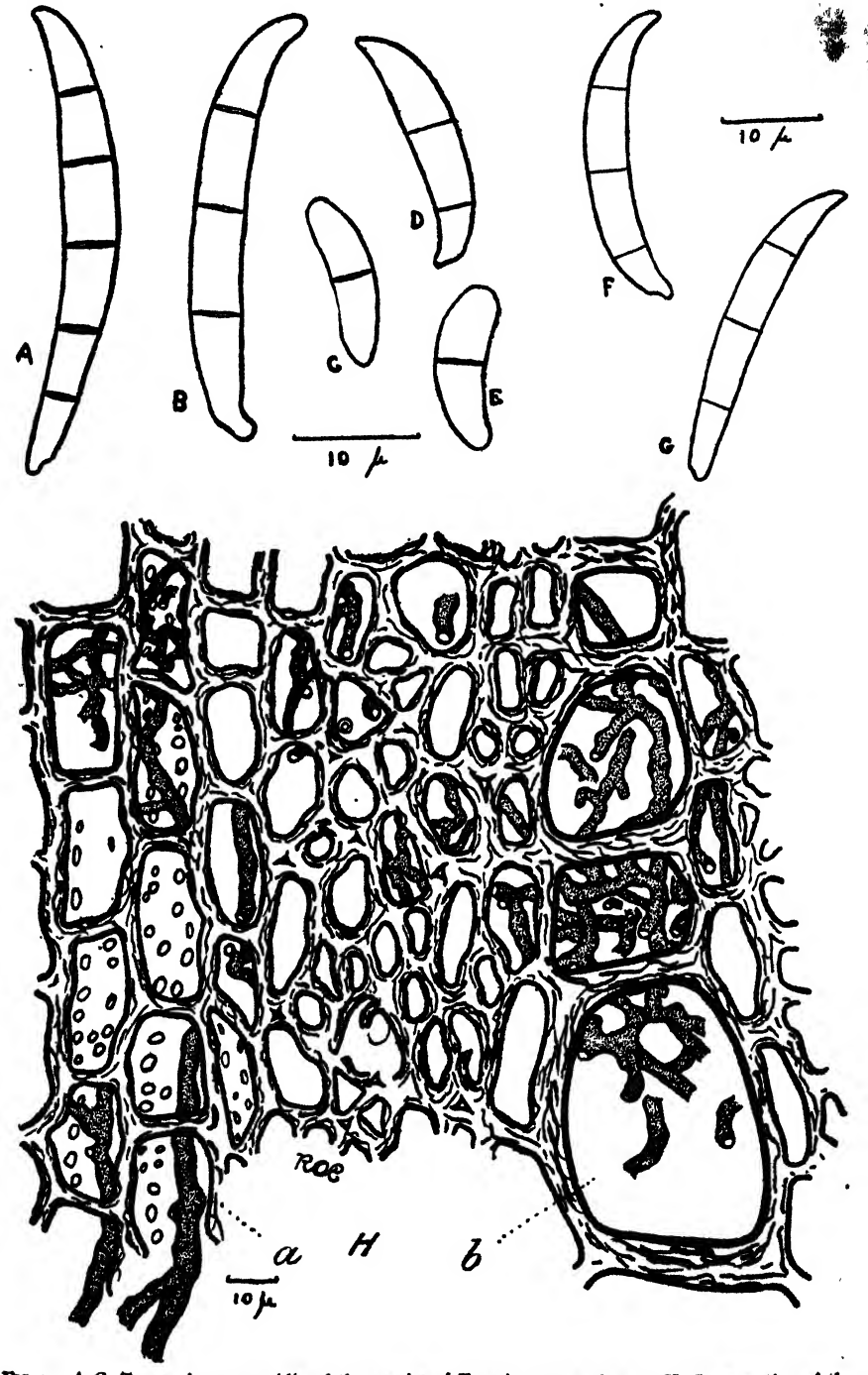

FIG. 1.—*A–G*, Types of macroconidia of the species of Fusarium on soybean. *H*, Cross section of the xylem portion of a diseased soybean stem, showing the invasion of the medullary rays (*a*) and the xylem vessels (*b*) by mycelia of the species of Fusarium on soybean.

and animals are also probably concerned in its spread. In all cases observed except one, nematodes have been present and probably facilitated the entrance of the fungus. The nematodes were found in infected sandy soil, but not in infected clay soil.

COMPARISON OF THE SOYBEAN SPECIES OF FUSARIUM WITH OTHER WILT-PRODUCING SPECIES OF THE GENUS

SOURCE OF CULTURES AND METHODS OF ISOLATION

Isolations were made from the interior of stems of freshly wilted soybean and cowpea plants. The stems were first thoroughly washed in water and allowed to remain wrapped in cotton moistened with 0.1 per cent solution of mercuric chlorid for 15 minutes. They were then split open so that the diseased interior was exposed. Fragments of diseased tissue were removed with a sterile scalpel and transferred to cooled poured plates of string-bean agar (8 c. c. per plate), to each of which four drops of 20 per cent lactic acid had been added. After several days, a microscopic examination was made of the conidia and mycelium to determine whether other organisms were present. Eight transfers to test-tube slants were made from the margin of several plantings and kept for comparison and for indications of contamination. It may be noted that a large percentage of pure cultures was obtained by this method. From the cultures that were pure, single-spore cultures were obtained according to the method described by Sherbakoff (20, p. 102–103; p. 104, footnote 8). Stock cultures were made from these single-spore cultures and repeatedly repoured to protect from subsequent contamination.

Several species of Fusarium were secured, in order to compare them with the *Fusarium* sp. from the soybean and the one from the cowpea, isolated as described above. The following species, subcultures from Wollenweber's authentic cultures, were obtained through the courtesy of Mr. C. W. Carpenter, of the Bureau of Plant Industry: *Fusarium oxysporum* (Schlecht.), *F. vasinfectum* (Atk.), *F. lycopersici* Sacc., *F. niveum* Smith (members of the section Elegans), and *F. discolor*, var. *sulphureum* (Schlecht.) App. and Wollenw. (1, p. 115–118), (section Discolor).

These species were studied in culture, in order to determine their morphological and cultural characters, since such a study is considered of primary importance in their differentiation. The species mentioned were chosen because all except one belong to the section Elegans, the section which contains the known wilt-producing species, and because, according to Wollenweber, they are the most difficult to separate by this method. *F. conglutinans* Wollenw., *F. redolens* Wollenw., and *F. orthoceras* App. and Wollenw., of the same section, are included in the comparisons. They are so different from the others, as indicated by the

original descriptions, that the writer soon realized that there was little probability of confusing them with the soybean strain. Wollenweber (25, 26) and Sherbakoff (20) have described other species and varieties of the section Elegans which are not, however, included in this study, because they occur on hosts widely separated genetically from the soybean [1] and because the authors have not had opportunity to make a sufficient number of infection experiments to establish them as wilt producers.

CULTURE MEDIA AND THEIR VARIOUS EFFECTS ON SPECIES OF FUSARIUM

In making a cultural study of these fungi much care was taken to follow the suggestions of Appel and Wollenweber (1), Wollenweber (25, 26), and Sherbakoff (20), in order to determine what criteria to employ in judging normal growth characters. It is generally believed that standardization of cultural methods is highly essential in the comparative study of so difficult a group of fungi.

The writer has kept the soybean and the cowpea strains under constant observation for two years on various kinds of "natural and artificial media" and under widely variable physical conditions. He is therefore familiar with the possible variability of members of this genus.

Since a large number of the media used did not prove to be of special diagnostic value, they are not discussed here. Among the media most commonly employed and serving some particular purpose were oat, potato, and string-bean hard agars (3 per cent agar), which, because of the paucity of moisture (20, p. 106), give all forms of fruitification with "normal" spores. Five to 10 per cent of dextrose was added to agars to favor the production of pigment. Growth on steamed rice in test tubes from weighed quantities of rice and measured amounts of water to obtain uniformity also results in the formation of pigment and sometimes an odor that is typical for certain related species of Fusarium. Herbaceous and woody stems, string-bean pods, and potato plugs give the best development of sporodochia and pionnotes.[2] Potato plugs also serve for the proper development of sclerotia and colors, both of which may be reduced or absent from stem plugs when there is a minimum development of mycelium.

According to Wollenweber (25, p. 37), virulence is commonly maintained on stem plugs. Living sterile soybean and cowpea seedlings grown in 6-inch test tubes were also used and are thought to be a better medium for maintaining virulence in the strains from the respective hosts.

[1] Wollenweber, H. W. (25, p. 37) says, "The parasite from one host, as a rule, has not been found on living organs of another host. In pure culture the parasite from one host . . . did not cause wilt in any other host as a result of inoculation experiments."

[2] For a discussion of these terms, see Wollenweber (25, p. 24, footnote).

In order to obtain sterile seedlings for this purpose the seeds were first washed for 5 minutes in tepid water and were then placed in concentrated sulphuric acid for 20 minutes. Formalin, mercuric chlorid, both in aqueous and alcoholic solution, and other disinfectants were employed with much less success. After washing off the acid in three or four changes of sterile water, the seed were transferred into sterilized moist chambers in the bottoms of which several layers of moist filter paper had been placed. Germinated seeds on which there was no evidence of contamination after a day or two were transferred to sterile test tubes [1] the bottom of each of which contained a wad of moistened filter paper.[2] If, during germination or transfer, contamination occurs, it generally becomes evident on the seedlings or white paper, especially if the seedlings are set aside until they have grown to a height of 3 or 4 inches.[3]

METHODS OF STUDY AND PRESENTATION

All transfers of different strains in a set for comparison were made to a certain medium on the same day and to additional media on later days until the set was growing on a sufficient number of media to provide the necessary cultural characters. When species were compared, they were always of the same age and were grown on the same medium. As many comparisons could be made on the same day as there were species and kinds of media in the set. If sufficient data had not been obtained, if certain cultures were abnormal, or if other species or media were to be used, new sets were prepared of all of the species using the desired media and comparisons were again made throughout the series.

Cultural differences also arise as a result of the employment of spores or a bit of mycelium in inoculation. In the former case the young cultures quickly produce spores with a scant mycelial growth, while in the latter the mycelial growth is abundant and there is a paucity of spores. For this reason spores from sporodochia, when present, were used, and in all cases, in so far as was possible, the same kind of inoculum was transferred for all cultures of a set. When the production of spores becomes subnormal, as it often does in cultures, considerable time and patience may be required to bring the strain back to a "*Normkultur*." This was accomplished by transferring a small portion of mycelium to a variety of media until a medium was found on which spores were again obtained.

[1] For making this last transfer, dip the ends of long tweezers into 95 per cent alcohol and ignite in the flame. This sterilizes instruments, burns off the excess of alcohol, and leaves them dry and cool enough for immediate use.

[2] The use of agar as a substratum for this purpose (Garman and Didlake, 7), and Sphagnum moss, did not prove to be satisfactory. Soil, too, has a disadvantage in that it does not show the contaminations as readily as filter paper or agar.

[3] An oat sprouter with glass front, heated by a kerosene lamp and costing about $10, makes a good light incubator for such purposes when the greenhouse is not conveniently located or the temperature suitable. This sprouter is unsuited, of course, to cultures or material requiring a constant temperature.

All cultures were kept in the laboratory at room temperature, 12° to 26° C., and in diffused daylight, so that they were subjected alike to any change of environmental conditions.

In all cases 10 cultures of a species were made on each medium. Different forms of fructification which normally appear on a certain medium may not do so in every tube. For example, in a species where sporodochia are not abundant, they may perhaps form on only 2 or 3 of the 10 stem plugs; or if the form produces green sclerotia, they may develop on not more than 5 of the 10 potato plugs. In some instances as many as 8 to 10 sets of 10 tubes each of a particular species were made.

In making the microscopical examination note was taken of the size, abundance, and type of conidia (fig. 1, *A-G*), chlamydospores, and conidiophores. In measuring spores several fields were first examined to fix in mind the prevailing type and an average of 10 or more of these typical spores was made. Careful note was taken also of extreme types.

In the macroscopic study of the cultures the nature of the stromata, the pionnotes and sporodochia, the character of the aerial mycelium, the color of spore masses, aerial and submerged mycelium and substratum, and the production of sclerotia were considered.

RESULTS OF THE COMPARISON OF THE SOYBEAN FUNGUS WITH OTHER MEMBERS OF THE SECTION ELEGANS

The first sets of parallel cultures were intended to serve in the separation of any or all of the species of Fusarium causing wilt from the soybean fungus. *F. discolor* var. *sulphureum*, *F. oxysporum*, *F. vasinfectum*, *F. lycopersici*, *F. niveum*, *F. tracheiphilum*, and *Fusarium* sp. from soybean were therefore grown on the following media, several sets of 10 cultures of each species being used on each medium: Potato plugs, steamed rice, cotton stems, potato hard agar, and string-bean hard agar. The cultures were examined when 8, 15, 19, 30, and 50 days old. The results are noted in Table I. Only those characters are recorded that are necessary or useful for the separation of the species.

TABLE I.—*Characters which separate a number of the wilt-producing species of Fusarium from F. tracheiphilum and the soybean fungus*

Species.	Sclerotia.	Sporodochia.	Pionnotes.	Chlamydospores.
F. discolor	None	Numerous	Perfect	Intercalary; no measurements.
F. vasinfectum	Green and flesh-colored.	do	do	Intercalary and terminal; no measurements.
F. oxysporum	do	Few	Reduced	Intercalary and terminal; 6 to 12 μ.
F. lycopersici	Flesh-colored	Numerous	Perfect	Intercalary and terminal; no measurements.
F. niveum	Large green	do	Reduced	Same as for *F. lycopersici*.
F. tracheiphilum	Green and flesh-colored.	Few	None	Intercalary and terminal; 6 to 12 μ.
Fusarium sp. on soybean	Mostly green; some flesh-colored.	do	do	Same as for *F. tracheiphilum*.

TABLE I.—*Characters which separate a number of the wilt-producing species of Fusarium from F. tracheiphilum and the soybean fungus*—Continued

Species.	Macroconidia.		Odor.
	Size of 3-septate.	Type.	
F. discolor	No data	Discolor; mostly 5-septate	None.
F. vasinfectum	Same as in *F. oxysporum*	Elegans; mostly 3-septate	Strong lilac on rice.
F. oxysporum	28.7 to 35.6 by 3.6 to 4.1 μ	do	Often none, sometimes scant lilac.
F. lycopersici	Abnormal	do	None.
F. niveum	Abnormal; (original description gives larger than in *F. Oxysporum*).	do	Do.
F. tracheiphilum	23.6 to 41.0 by 3.9 to 4.1 μ	do	Do.
Fusarium sp. on soybean	24.6 to 35.8 by 2.89 to 4.1 μ	do	Do.

From the data in Table I it is important to observe that *F. tracheiphilum* and the species of Fusarium on soybean belong to the section Elegans, as established by Appel and Wollenweber (1) and modified by Wollenweber (25) in a subsequent study. They are themselves very similar in cultural characters, but can be quite sharply separated from the other species included in the tabulation. When the characters of the species of Fusarium on the cowpea and soybean noted in this table are compared with those in the original descriptions of certain other members of the section Elegans—namely, *F. redolens*, *F. orthoceras*, and *F. conglutinans*, there is plainly no chance of their confusion. *F. redolens* (25) produces no blue sclerotia, and its conidial masses are brownish white; *F. orthoceras* (25) possesses neither sclerotia, sporodochia, nor pionnotes; and *F. conglutinans* (25) is distinguished because of the absence of the typical wine-red to purple colors of the section.

MORPHOLOGICAL AND CULTURAL COMPARISON OF THE FUSARIUM SP. ON SOYBEAN WITH F. TRACHEIPHILUM

Since the studies summarized in Table I do not succeed in distinguishing the species of Fusarium on soybean and cowpea, a more extensive cultural study of these two fungi was made. For this purpose three series of cultures were grown, and the results have been summarized in Table II. Each series contained 10 cultures of each fungus on stem plugs, potato plugs, steamed rice, standard nutrient agar (1.8 per cent agar and 1.0 per cent acid), string-bean hard glucose agar (3 per cent agar, 1.0 per cent acid, and 10 per cent glucose), and oat hard agar (3 per cent agar and 1.0 per cent acid). The cultures were examined when they were 8, 15, 30, 50, and 75 days old.

TABLE II.—*A morphological comparison of the species of Fusarium on soybean and cowpea*

FUSARIUM SP. ON SOYBEAN

Medium.	Macroconidia.	Sporodochia.	Sclerotia.	Color of mycelium.	Character of mycelium.
Standard nutrient agar.	No measurements.	Salmon-colored..	None.....	White.....	Mostly aerial and floccose, becoming appressed in old age.
String-bean agar.....	.....do.....	Salmon-colored; generally present.	Green.....	.....do.....	Do.
Oat hard glucose agar.	26.6 to 38.6 by 3.69 to 4.92 μ 50 days old.	Flesh-colored....	Dark green..	Mostly lilac; some dark purple.	Cottony.
Steamed rice.....	.....	.....	.....	Reds, pinks, lilacs, purples.	
Potato plugs.....	Normal spores absent.	Salmon-colored; generally present on sclerotia.	Dark green..	Green near sclerotia.	Floccose.
Stem plugs.....	22.5 to 43.6 by 2.87 to 4.11 μ 14 days old.	Salmon-colored; small.	Green; very small; numerous.	White; sometimes green near sclerotia.	Floccose; scant.

F. TRACHEIPHILUM

Standard nutrient agar.	.....	None....	Flesh-colored..	White.....	Mostly submerged or appressed.
String-bean agar.....	No measurements.	Salmon-colored; few.	Mostly flesh-colored; some green.	.....do.....	Do.
Oat hard glucose agar.	22.5 to 36.9 by 3.8 to 4 42 μ 50 days old.	Flesh-colored..	Dark green and flesh-colored.	Mostly dark purple; some lilac.	Cottony to matted and appressed.
Steamed rice.....	.....	.....	....	Pinks, reds, lilacs, purples.	
Potato plugs.....	24.6 to 36.9 by 3.28 to 4.42 μ 19 days old.	Salmon-colored; often on sclerotia.	Flesh-colored; often none.	Pinks, lilacs, greens.	Mostly appressed.
Stem plugs.....	.....	Salmon-colored; small; sometimes none.	Green; very small; numerous.	White; sometimes green near sclerotia.	Appressed good growth

No mention is made in Table II of pionnotes or odors, as none were produced in any of the cultures. The microconidia of both strains show a wide variation both in size and shape, but these differences can properly be included in the range of variation. The normal macroconidia of the soybean (fig. 1, *A–G*) and cowpea strains are indistinguishable. The chlamydospores of either strain are terminal or intercalary in or on vegetative filaments and average 6 to 10.25 μ in diameter. The conidiophores are verticillately branched when normal. Sporodochia, although sometimes flesh-colored, are normally salmon-colored. They are not always present on all media, but are formed by each strain either on sclerotia or on mycelia as stromatal bases. Green sclerotia are normally present in both strains. There appear to be some differences in colors produced in substrata, although not very consistent ones, a difference in the character of mycelium until advanced ages of the cultures and gen-

erally, but not always, an absence of flesh-colored sclerotia in the soybean fungus. These differences, however, are not believed to be of sufficient importance to warrant regarding the soybean strain as a distinct species or variety.

In addition to the media employed in Table II, potato hard agar, cornmeal plugs, and string-bean pods were used; but they showed no additional characters of value.

Perithecia have never been observed on the diseased stems; neither have they been obtained in cultures from the surface spores nor from the diseased internal tissues. In fact, the cultural differences between the *Fusarium* sp. on soybean and *Neocosmospora* spp. are as striking as between *Neocosmospora* spp. and the several species of Fusarium causing wilt studied by Higgins (12) and Butler (3).

INOCULATION EXPERIMENTS

From the foregoing morphological and cultural studies, it is evident that the species of Fusarium on soybean is not distinguished from *F. tracheiphilum* by any well-defined differences. Since the possibility existed that they might be separated by biological differences, reciprocal inoculation studies were undertaken to secure additional evidence of their identity.[1]

Plants were therefore grown in pots and flats in the greenhouse and in plots in the field for use in inoculations. The soil used in the pots and flats was a fine, compact, sandy loam, except in the case of one experiment, and was taken from a field in which diseases of cowpeas and soybeans caused by *Fusarium* spp. had never been observed. In certain of these tests, as an added precaution, the soil was partially sterilized by the use of a 2 per cent solution of formaldehyde. The seed were also sterilized in certain experiments by immersion for 15 minutes in commercial sulphuric acid. Since uninoculated plants remained free from disease when these precautions were not employed, their use was discontinued in subsequent tests.

The pots and flats were of sufficient size to permit the plants to grow to maturity.

In determining the percentage of diseased plants, count was made only of those in which it was possible to find discoloration and invasion of the xylem tissues. In case of doubt in this microscopic examination, planted plates were made from the tissues and the subsequent growths studied.

The varieties of soybeans and cowpeas planted for the cross-inoculation experiments were known to be subject in the field to the species of Fusarium on soybean and cowpea, respectively.

EXPERIMENT I.—Twenty-five North Carolina Black cowpea and 25 Mammoth Yellow soybean seedlings, growing in each of two flats

[1] Wollenweber (25, p. 37) says that a consideration of the biological characters is of secondary importance in the determination of species.

in the greenhouse, were each inoculated when from 3 to 6 inches high with spores from sporodochia and with mycelium by introducing the material into incisions in the stems an inch or two below the surface of the soil. All of the plants in one flat were inoculated with the soybean strain of Fusarium and all of those in the other with the cowpea strain. Checks and all inoculated plants except two cowpeas inoculated with the soybean strain and one with the cowpea strain, remained healthy. The test was repeated, using a freshly isolated strain of both organisms; and, since all but one of the plants remained healthy, this method of inoculation was discarded.

EXPERIMENT II (Series 1).—In this experiment the soil in two flats A and B, in the greenhouse was inoculated with pure cultures of *Fusarium* spp. on cowpea and soybean, respectively. These cultures were then incorporated in the upper 4 inches of soil.

The organisms had been grown on pieces of moistened, sterilized cowpea stems until numerous sporodochia had formed. On April 12, 1916, 20 North Carolina Black cowpeas and 20 Mammoth Yellow soybeans were planted in each flat. A third flat, containing uninoculated soil, was planted as a check.

By June 4 a cowpea plant in flat B was noted to be diseased. Others had been observed to be affected by June 15, when all the plants were removed and examined. The results are presented in Table III.

TABLE III.—*Results of growing soybeans and cowpeas in artificially inoculated soil*

Flat.	Organism.	Host.	Total number of plants.	Diseased plants.	
				Number.	Percentage.
A	*Fusarium* sp. on cowpea	Cowpeas	20	6	30
		Soybeans	20	3	15
B	*Fusarium* sp. on soybean	Cowpeas	20	10	50
		Soybeans	20	7	35
C	None (control)	Cowpeas	20	0	0
		Soybeans	20	0	0

EXPERIMENT II (Series 2.)—Since the percentage of diseased plants in series 1 is relatively small, the test was repeated, using another strain of each organism and Clay cowpeas instead of the North Carolina Black variety. Each plant in this test was injured by incision below the surface of the inoculated soil. The period of growth of these plants extended from July 29 to September 1, at which date the plants were fully matured. The results of this series are recorded in Table IV.

EXPERIMENT II (Series 3).—The test in series 2 was duplicated between September 7 and November 20, with no resultant increase in the percentage of infections.

EXPERIMENT III.—Since it was thought that the strain of Fusarium on soybean had to a degree lost its virulence by growth in culture, soy-

bean stems bearing an abundance of sporodochia were macerated and mixed with the soil in two flats. Seed of the Mammoth Yellow variety were planted on May 25. When the experiment was concluded, August 10, only 8 of the 80 soybean plants in these two flats were found to be infected.

TABLE IV.—*Results of growing soybeans and cowpeas in artificially inoculated soil, the plants having been wounded below the surface of the soil*

Flat.	Organism.	Host.	Total number of plants.	Diseased plants.	
				Number.	Percentage.
D	*Fusarium* sp. on cowpea.....	Cowpeas.	20	3	15
		Soybeans...	20	3	15
E	*Fusarium* sp. on soybean..... ...	Cowpeas .	20	6	30
		Soybeans.	20	5	25
F	None (control).....	Cowpeas.	20	0	0
		Soybeans .	20	0	0

EXPERIMENT IV.—This experiment was made with soybeans between September 26 and December 1 in an attempt to determine whether the presence of nematodes increases the number of infections. The nematodes were introduced into the soil of large buried pots in root galls from living soybeans free from infection by *Fusarium* sp. The results are presented in Table V.

TABLE V.—*Influence of nematodes on the percentage of infection of soybeans with species of Fusarium*

Organism.	Total number of plants.	Number with nematode galls.	Number with *Fusarium* sp.
Fusarium sp. on cowpea and nematodes.....	10	10	2
Fusarium sp. on cowpea without nematodes	10	0	0
Fusarium sp. on soybean and nematodes.	10	10	3
Fusarium sp. on soybean without nematodes.. . . .	10	0	2
Nematodes only.....	10	10	0
None (control).....	20	0	0

Only one test of this kind was made; and it is significant to note that there was no increase in infection by the fungus, although the plants in all of the pots into which the galls had been introduced were attacked by eelworms. This experiment was concluded before the plants had matured.

EXPERIMENT V.—Since the more porous types of sandy soil have generally been observed to favor the development of *Fusarium* spp., the cause of wilt diseases, an experiment was performed which was in duplication of Experiment II, series 1, except that the soil consisted of a mixture of three parts of medium-coarse sand and one part of fine sandy

loam and stable manure. The results obtained between September 7 and the close of the experiment, November 20, show considerable increase in the percentage of infection over those in the more compact, fine sandy loam of the preceding experiments even though the cultures used were transfers from cultures nearly 3 months old. Cultures of the strain of *Fusarium* on cowpea were added to flat G, of the species of *Fusarium* on soybean to flat H, and flat J was held as a control. Table VI contains the data on this experiment.

TABLE VI.—*Influence of soil on percentage of infection by Fusarium spp.*

Flat.	Organism.	Host.	Total number of plants.	Diseased plants.	
				Number.	Percentage.
G	*Fusarium* sp. on cowpea	Cowpeas	20	16	80
		Soybeans	20	12	60
H	*Fusarium* sp. on soybean	Cowpeas	20	13	65
		Soybeans	20	12	60
J	None (control)	Cowpeas	20	0	0
		Soybeans	20	0	0

EXPERIMENT VI.—This experiment was designed to confirm the results of inoculations in the greenhouse by inoculations under partially controlled field conditions. Four small plots (No. 26, 27, 28, and 29) on wilt-free soil of the station farm were inoculated as in the previous experiments with pure cultures of the species of Fusarium on soybean; plots No. 59 and 60 were inoculated with the *Fusarium* sp. on cowpea; and two others (100 and 101) were left untreated as controls. Thirty cowpeas and thirty soybeans were planted in each plot on June 10, and the final results noted in Table VII were obtained on September 4.

TABLE VII.—*Results of cross-inoculations in the field*

Plot No.	Organism.	Host.	Total number of plants.	Diseased plants.	
				Number.	Percentage.
26	*Fusarium* sp. on soybean.	Clay cowpeas	30	17	56.6
		Haberlandt soybeans	30	4	13.3
27	.....do.....	Clay cowpeas	30	10	33.3
		Tokio soybeans	30	0	0.0
28	....do.....	Clay cowpeas	30	10	33.3
		Mammoth Yellow soybeans	30	8	26.6
29	.....do.....	Clay cowpeas	30	15	50.0
		Tar Heel Black soybeans	30	3	10.0
59	*Fusarium* sp. on cowpea.	Clay cowpeas	30	26	86.6
		Tokio soybeans	30	6	20.0
60	....do.....	Clay cowpeas	30	17	56.6
		Mammoth Yellow soybeans	30	6	20.0
100	None (control)	Clay cowpeas	30	0	0.0
		Mammoth Yellow soybeans	30	0	0.0
101	.....do.....	Clay cowpeas	30	0	0.0
		Mammoth Yellow soybeans	30	0	0.0

EXPERIMENT VII.—On May 25, 1916, two 100-foot rows of each of the soybean varieties Tokio, Haberlandt, Mammoth Yellow, Medium Yellow, and Virginia were planted in a field which produced a large percentage of wilt in cowpeas in 1914. Two rows of cowpeas were planted in the same plot. By September 1, when all the plants had fully matured, a small percentage of wilted cowpeas had been noted; but no blighted soybeans were found.

Similar data were obtained from observations on cowpeas and soybeans grown in the experimental plot devoted to plant breeding. In this 4-acre plot, three or four rows of soybeans were alternated with three or four rows of cowpeas throughout the field. Some wilt occurred in practically every row of cowpeas in the plot, but careful examinations during the season failed to reveal a single soybean blighted with *Fusarium* sp. among 17 standard varieties and 50 other unnamed selections.

EXPERIMENT VIII.—The field at Red Springs, N. C., in which at least 60 per cent of the Mammoth Yellow soybeans were blighted in 1915, was again planted with this variety on May 23, 1916. In a portion of the field which had been reserved for the purpose, one 54-meter row each of Haberlandt, Mammoth Yellow, Pekin, Black Eyebrow, Medium Yellow, and Tar Heel Black soybeans and a row of Clay cowpeas were planted on June 8. On August 10 the main field and all of the varieties in the test, including the cowpeas, showed considerable blight or wilt, except the Black Eyebrow and the Virginia varieties of soybeans. On August 26 the latter of these varieties was apparently free from disease, but the plants had declined with age to such an extent that the exact determination was doubtful. The Black Eyebrow variety, however, remained free from disease throughout the season.

SUMMARY

(1) A disease of soybeans not previously reported has been studied during the past two years.

(2) The disease is characterized by a chlorosis and shedding of the leaves or leaflets, followed by the death of plants, and is herein called "blight or wilt."

(3) Soybean-blight has been observed in several localities within North Carolina on soils infected with cowpea-wilt.

(4) A species of Fusarium belonging to the section Elegans is the causal organism.

(5) Cultural and morphological studies which are regarded as of primary importance in distinguishing species of Fusarium show that the strain of Fusarium on soybean is identical with the organism producing the wilt of cowpeas.

(6) Reciprocal inoculation experiments with the strains from soybeans and cowpeas show that cross-inoculations can be made. These experiments were conducted in the greenhouse and under field condi-

tions. Pure cultures of the two strains were used in certain of the experiments and inoculum from the natural host in others.

(7) Infection probably occurs through the roots, but nematodes appear not to increase the percentage of blight materially.

(8) The character of the soil appears to influence the percentage of infection, since the largest proportion of diseased plants appeared in coarse sandy soil.

(9) Blight or wilt of soybeans is therefore due to *Fusarium tracheiphilum* Smith.

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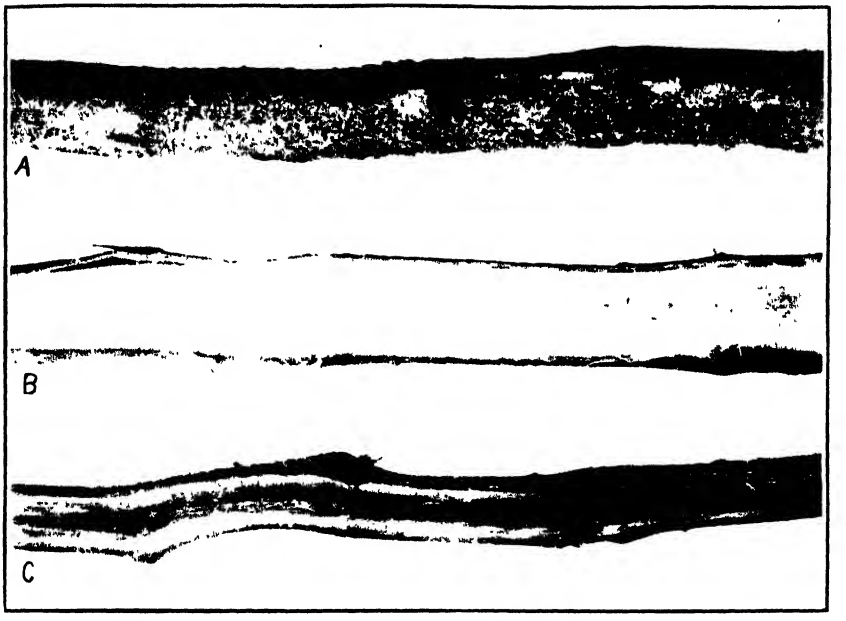
PLATE 95

A.—A diseased stem of soybean, showing the roughened appearance caused by the irregular covering of sporodochia.

B.—Interior of healthy (unstained) stem of soybean.

C.—Interior of diseased (discolored) stem of soybean.

D.—Soybean plants grown out of doors in the same type of clay soil: *D*, healthy; *E*, diseased through the naturally infected soil.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PEANUT-WILT CAUSED BY SCLEROTIUM ROLFSII

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INTRODUCTION

Wolf [1] records a fruitrot of peanuts (*Arachis hypogaea*) caused by *Sclerotium Rolfsii* Sacc., but states (p. 143) that "usually there is no indication of the disease in the appearance of the aboveground parts." During the summer of 1915 the writer observed wilted peanut plants on two plots of peanuts at the Virginia Truck Experiment Station, at Norfolk, Va. The associated organism was found to be *S. Rolfsii*, and its pathogenicity has been established.

Observations are here presented on the life history of the causal organism and experiments on control measures by the use of resistant varieties of peanuts during the season of 1915 and 1916. Data relative to the relation of *S. Rolfsii* to other crops used on the experimental plots are also given.

HISTORY OF THE DISEASE

In 1910 an experiment was started at the Virginia Truck Experiment Station to determine the value of rotation as compared with the continuous planting of peanuts. This experiment, which is still being conducted, occupies four adjoining plots, each about one-third of an acre in size.

Plots 1 and 2 were planted to peanuts in 1910. The next year plots 1 and 3 were planted to peanuts, while plots 2 and 4 were planted to the other crops used in the rotation. In 1912, plots 1 and 4 were in peanuts, the other two being planted to the other rotation crops. In 1913, the 3-year rotation had been completed, so peanuts again occupied plots 1 and 2.

The source of the peanut seed used in beginning this experiment was not known, but during the years 1911 and 1912 peanuts from the previous year's crop were used for seed.

Table I shows the position of the four plots, with their respective crops for the years 1910 to 1916, inclusive.

[1] Wolf, F. A. Leaf spot and some fruit rots of peanut. Ala. Agr. Exp. Sta. Bul. 180, p. 127–130, 5 pl. 1914.

TABLE I.—*Crops grown on plots 1 to 4 from 1910 to 1916, inclusive*

Year.	Plot 1 (continuous planting).	Plot 2 (rotation).	Plot 3 (rotation).	Plot 4 (rotation).
1910	Peanuts....	Peanuts.....	Cowpeas, fall potatoes.	Corn, crimson clover.
1911	.....do.....	Corn, crimson clover.	Peanuts.....	Cowpeas, fall potatoes.
1912	.....do.....	Cowpeas, fall potatoes.	Corn, crimson clover.	Peanuts.
1913	.....do.....	Peanuts.....	Cowpeas, fall potatoes.	Corn, crimson clover.
1914	.....do.....	Corn, crimson clover.	Peanuts.....	Cowpeas, fall potatoes.
1915	.....do.....	Cowpeas, fall potatoes.	Corn, crimson clover.	Peanuts.
1916	.....do.....	Peanuts.....	Cowpeas, fall potatoes.	Corn, crimson clover.

From Mr. W. R. Beattie, formerly of the Office of Horticultural Investigations of the United States Department of Agriculture, it was learned that peanut leafspot, caused by *Cercospora personata*, was the only disease observed during the years from 1910 to 1912.

In 1913, a fresh supply of peanut seed of the Valencia variety was obtained from a concern in Norfolk which buys peanuts in large quantities from various States for making peanut butter and confections. The seed for each succeeding year was saved from peanuts grown on the experimental plots the previous year.

It was learned that a few wilted peanut plants were observed in the 1913 crop and that the number of diseased plants increased each year.

In 1916, the writer planted in experimental plots 1 and 2 Valencia peanuts which were obtained from the Department of Agriculture. Some of these seeds were also planted in a plot near by which had not grown peanuts for at least several years. Over 15 per cent of the plants in the experimental plots wilted, while all of the plants in the plots near by remained healthy. These data, together with the observation of the wilted plants in 1913 and each succeeding year, lead the writer to conclude that the wilt-producing organism was not present in the soil previous to 1913, but had been introduced with the fresh supply of seed used that year.

DESCRIPTION OF THE DISEASE

Under field conditions in Virginia this peanut-wilt begins to show when the plants are between 1 and 2 months old and continues to develop on new plants, here and there, throughout the season. In some cases one or two shoots wilt, while the rest of the shoots appear healthy, but in the majority of cases the whole plant eventually wilts, as shown in Plate 96, *A*. Examination of the wilted shoots generally discloses coarse white mycelium and small brownish sclerotia about the size of

mustard seed near the base of the wilted shoots (Pl. 96, *B*). On sectioning the basal portion of diseased shoots it is found that the tissues are discolored and decaying, indicating that the wilting of these shoots is caused by cutting off the water supply near the base.

Abundant white mycelium and brownish sclerotia also developed on peanut leaves which dropped to the soil about the base of wilted plants. Plate 97, *A*, shows peanut leaflets taken from the surface of the soil under a wilted plant. Thirty-seven sclerotia have been counted on a single leaflet; and in many cases sclerotia developed in greater numbers about the base of wilted shoots, thus providing ample means for reproducing and spreading the organism in subsequent seasons.

Although not as commonly observed, the coarse white mycelium and sclerotia may also be found on both the outside and inside of pods on wilted plants.

During the seasons of 1915 and 1916 wilted plants were observed scattered over the entire area of each plot, indicating that the organism was well distributed throughout the soil. In some cases, however, the plants in adjoining hills wilted in a progressive manner, as though the fungus spread from centers of infection. Plate 97, *B*, shows three such hills, the one in the foreground being infected first. The fungus then apparently spreads to the second hill 12 inches distant, and then to the third hill, where a portion of one plant had been attacked and had wilted at the time the photograph was taken.

SERIOUSNESS OF THE DISEASE

As a result of counts made in 1915 it was found that 15 per cent of the plants had one or more wilted shoots. Similar counts in 1916 showed that the percentage of plants having one or more wilted shoots had increased.

Shoots attacked early in the season produce no peanuts, and those infected after peanuts have formed generally become so badly diseased that the fruits fail to mature. The diseased shoots are so withered and shrunken that they are of little or no value for hay; therefore, wilted plants are practically a total loss.

PATHOGENICITY AND IDENTITY OF THE ASSOCIATED ORGANISM

As coarse white mycelium and small brownish sclerotia were constantly associated with the wilted plants, it seemed advisable to obtain pure cultures of this fungus for identification. Basal sections from numerous diseased shoots were externally disinfected in alcohol and in mercuric chlorid (1:1,000) and plated in nutrient agar. In five days coarse white mycelium had grown out from most of the sections. Transfers of this mycelium to tubes of corn meal and bean pods grew rapidly and eventually produced brownish sclerotia similar to but somewhat larger than those found on the wilted peanut plants. Other fungi grew out from

some plated sections of wilted shoots, but none of these had white mycelium, and all failed to produce the brownish sclerotia.

Healthy peanut plants of various ages were inoculated by placing pure cultures of the various fungi in contact with normal and with wounded stems just below the surface of the soil. Similar plants were labeled to serve as checks. The plants inoculated with the white mycelium and sclerotia began to wilt within a period varying from 15 days to 2 months after inoculation, while the control plants remained healthy. White mycelium and brownish sclerotia developed at the base of artificially infected plants. The causal organism was reisolated from the wilted stems and found to be the same as the original isolations. Plants inoculated with various other fungi isolated from diseased peanut shoots remained healthy.

Isolations of the causal organism were made from time to time during the seasons of 1915 and 1916, and the fungus producing the coarse white mycelium and brownish sclerotia was found to be constantly associated with the wilted peanut shoots.

The appearance of the peanut-wilt fungus corresponded so closely to descriptions of *S. Rolfsii* that the writer tentatively identified the fungus as this organism, although he had not seen any report of this fungus being found in Virginia. The fact that Wolf[1] had, in 1914, reported *S. Rolfsii* as being the cause of a fruitrot of peanuts in Alabama added weight to the writer's suspicion that the peanut-wilt organism found in Virginia is *S. Rolfsii*. During the winter of 1915–16 cultures of the peanut-wilt organism were compared with stock cultures of *S. Rolfsii* from two authentic sources, and the organisms were found to be identical as regards mycelial development, and the size, shape, and color of the sclerotia, thus indicating that this peanut-wilt fungus is *S. Rolfsii*. In January, 1916, the opportunity was afforded the writer to examine specimens of peanut stems infected with *S. Rolfsii* which had been collected at Lykesland, S. C., in 1909 by Dr. W. A. Orton, of the United States Department of Agriculture. They were similar to specimens collected in Virginia by the writer during the season of 1915 and convinced him that the Virginia peanut-wilt is caused by *S. Rolfsii*.

IMPORTANCE OF SEED AS A MEANS OF SPREADING SCLEROTIUM ROLFSII

Having observed that peanut pods from diseased plants bore mycelium and sclerotia of *S. Rolfsii* both externally and internally, the writer deemed it advisable to test the importance of seed and its accompanying trash as a means of transmitting the peanut-wilt fungus.

Seed for the bulk of the 1916 planting was hand-shelled from the 1915 crop by laborers who made no attempt to discriminate between good

[1] Wolf, F. A. Op. cit.

and bad seed. On the same day that the above seed was planted by the laborers the writer planted in each plot one row of seed which he had carefully selected from healthy pods of the 1915 crop. Notes were taken throughout the season, and it was found that the rows planted to carefully selected healthy seed had as many wilted plants as the average of the rows planted with unselected seed. The writer does not think that these data prove that seed is unimportant in spreading *S. Rolfsii*, but it does indicate that the soil of both plots was well infested with this organism. The writer anticipates conducting further experiments along this line during the season of 1917.

From what has been observed of the hand-shelling of peanuts for seed, it seems reasonable to suspect that *S. Rolfsii* may be carried by sclerotia and bits of infected pods which become mixed with the shelled seed. This danger would be greatly lessened by fanning the shelled seed to remove the trash, or by obtaining heavy clean seed from a reliable storage plant which has suitable facilities for thorough cleaning.

EFFECT OF ROTATION ON THE DISEASE

With many fungus diseases a 3-year rotation would be effective in reducing the amount of disease on the susceptible crop, but from the data in Table II it will be seen that this is not true in the case of peanut-wilt.

By reference to Table I, it will be seen that in 1916 peanuts were again planted on plots 1 and 2, which were the plots planted to peanuts in 1913, the year when the peanut-wilt was first observed. Since 1913, plot 1 has been continuously in peanuts, while plot 2 has grown crops of corn and crimson clover in 1914 and a fall crop of Irish potatoes in 1915. When again planted to peanuts in 1916, plot 2 had a somewhat larger percentage of wilted plants than plot 1. There is a possibility that the wilt in plot 2 in 1916 was due to infection introduced with the seed and trash; but the fact that healthy Valencia seed planted by the writer in these two plots also showed a high percentage of disease would indicate that a rotation of more than three years is necessary to free a soil from *S. Rolfsii*.

VARIETAL RESISTANCE TO SCLEROTIUM ROLFSII

Through the courtesy of Mr. F. E. Miller and Mr. H. C. Thompson, of the Office of Horticultural Investigations, United States Department of Agriculture, the following varieties of peanuts were obtained: Tennessee Red, Valencia, Spanish, African, Virginia Bunch, Virginia Runner, and Hog Goober (*Worandzia subterranea*).

To serve as controls, one row of each of these varieties was planted on land which had not previously been planted to peanuts. To test their varietal resistance to *S. Rolfsii*, from a half to a full row of the different varieties was planted in each plot, using every sixth row. These seed

were planted at the same time as the Valencia seed used in the rotation experiment, and the resulting plants were all given the same treatment as regards cultivation. Notes were taken throughout the season and the results are given in Tables II and III.

TABLE II.—*Resistance of various varieties of peanuts to Sclerotium Rolfsii compared with Station Valencia seed on adjoining rows on plot 1*

Variety.	Row.	Number of hills.	Number of wilted hills.	Variety.	Row.	Number of hills.	Number of wilted hills.
Station Valencia	5	66	7	Station Valencia	17	66	11
Government Valencia	6	66	7	Virginia Bunch	18	66	6
Station Valencia	7	66	11	Station Valencia	19	66	8
Do	8	66	7	Do	17	66	10
Tennessee Red	6	66	2	Virginia Runner	18	66	.....
Station Valencia	7	66	11	Station Valencia	19	66	8
Do	11	66	14	Do	23	132	7
Spanish	12	66	7	African	24	130	.....
Station Valencia	13	66	16	Station Valencia	25	132	12
Do	11	66	14				
Hog Goober	12	40	.....				
Station Valencia	13	66	13				

From the data it is seen that Valencia peanuts from Station seed and from Government seed are about equally susceptible to *S. Rolfsii*. Tennessee Red peanuts are considerably more resistant than Valencia. Spanish peanuts are slightly less susceptible than Valencia. Hog Goober is practically immune to *S. Rolfsii*. Virginia Bunch is more resistant than Valencia. Virginia Runner is practically immune. African is practically immune.

TABLE III.—*Resistance of varieties compared with Station Valencia on adjoining rows in plot 2*

Variety.	Row.	Number of hills.	Number of wilted hills.	Variety.	Row.	Number of hills.	Number of wilted hills.
Station Valencia	5	132	29	Station Valencia	17	132	17
Government Valencia	6	132	20	Spanish	18	140	15
Station Valencia	7	132	31	Station Valencia	19	132	29
Do	11	66	14	Do	23	66	13
Tennessee Red	12	66	2	Virginia Bunch	24	66	.....
Station Valencia	13	66	8	Station Valencia	25	66	15
Do	11	66	14	Do	23	66	12
African	12	66	.....	Virginia Runner	24	66	.....
Station Valencia	13	66	8	Station Valencia	25	66	14

The results obtained on plot 2 and presented in Table III substantiate those of plot 1, and indicate that the African, Virginia Runner, Hog Goober, Virginia Bunch, Tennessee Red, and Spanish varieties are resistant to *S. Rolfsii* about in the order named, and all are more resistant than Valencia.

Thompson and Bailey [1] classify Valencia and Tennessee Red peanuts as the same variety, but the writer noted sufficient difference in resistance to *S. Rolfsii* to make it advisable to consider these two as separate varieties from a pathological viewpoint.

As the Virginia Runner, Virginia Bunch, and Spanish varieties are the three varieties grown in Virginia, there is little to be feared from the attacks of *S. Rolfsii* in commercial fields in this State. If, however, this organism should get established in a soil used for peanuts, it would seem advisable to grow resistant varieties in preference to carrying on a rotation of more than three years in order to starve it out.

EFFECT OF SCLEROTIUM ROLFSII ON CRIMSON CLOVER USED AS A COVER CROP

Wolf [2] mentions cowpeas and crimson clover as succumbing to artificial inoculations with *S. Rolfsii*. The crimson clover grown for a winter cover crop on the experimental peanut plots at Norfolk is generally planted in September or October and turned under in May. Under these field conditions in Virginia the writer has observed no infection of crimson clover during 1915 and 1916.

SUMMARY

(1) In 1915 a wilt of peanut plants was found in experimental plots at the Virginia Truck Experiment Station.

(2) The causal organism was probably introduced into the plots in 1913 with a fresh supply of Valencia seed imported that year.

(3) The disease under field conditions appeared when the plants were from 1 to 2 months old and continued to develop throughout the season.

(4) The wilting was due to the fungus attacking the shoots at or near the surface of the soil and killing the invaded tissues. White mycelium and brownish sclerotia about the size of mustard seed were found about the base of wilted peanut stems and also on peanut leaves which had dropped to the soil about the base of wilted plants. Mycelium and sclerotia were also found associated both externally and internally on the pods on diseased plants.

(5) Wilted plants were scattered over the entire area of each plot, thus indicating that the causal organism was well distributed. In some cases several adjoining hills were observed to wilt in a progressive way, which indicated the spreading of the fungus from one hill to the one adjoining in the same row.

(6) Isolations from diseased shoots and inoculations with pure cultures of the associated organisms established the pathogenicity of the fungus producing the coarse white mycelium and brownish sclerotia. Comparisons of pure cultures of this fungus with descriptions and stock

[1] Thompson, H. C., and Bailey, H. S. Peanut oil. U. S. Dept. Agr. Farmers' Bul. 751, 16 p. 1916.
[2] Wolf, F. A. Op. cit., p. 144–145.

cultures of *Sclerotium Rolfsii* established the identity of the peanut-wilt fungus as this organism.

(7) Counts of wilted plants in 1915 showed that 15 per cent had one or more diseased shoots. Similar counts in 1916 showed a larger percentage of wilted plants. No fruits are produced by shoots attacked early in the season, and shoots attacked later in the season generally fail to mature their fruits. Diseased shoots are dry and shrunken and of little or no value for hay.

(8) Preliminary tests indicated that the careful selection of healthy seed did not reduce the amount of wilt; probably due to the fact that the soil was thoroughly infested with *S. Rolfsii*.

(9) Data relative to the value of a 3-year rotation indicate that *S. Rolfsii* lives over in the soil for three years and produces as large a percentage of disease on land where a 3-year rotation was practiced as on land continuously planted to peanuts.

(10) Tests of six varieties of peanuts and one hog goober, *Worandzia subterranea*, planted in soil badly infested with *S. Rolfsii* showed that the Valencia peanut is most susceptible to this organism and that Spanish, Tennessee Red, and Virginia Bunch are respectively resistant in the order named, while Virginia Runner, African, and Hog Goober are practically immune to *S. Rolfsii*.

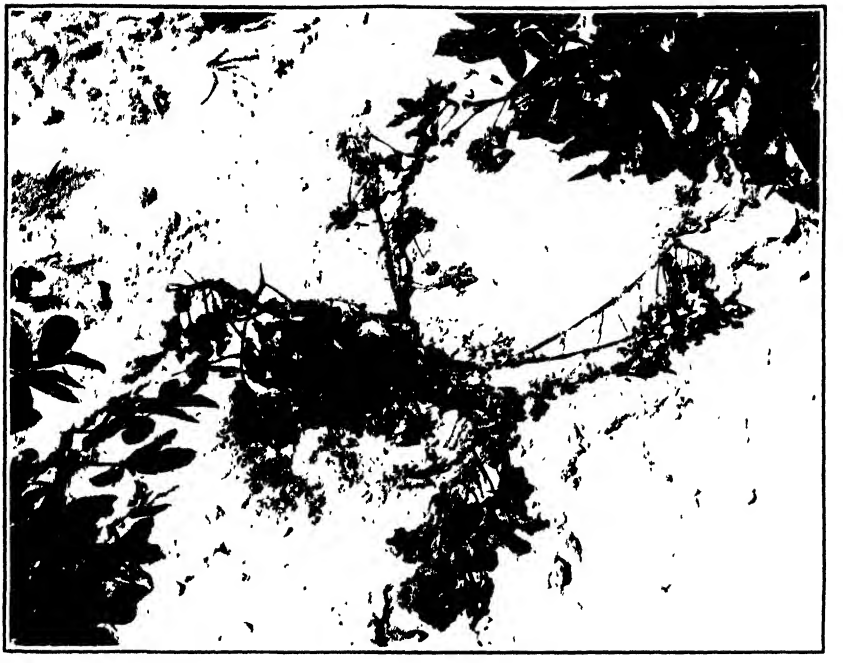
(11) Experiments indicate that soil infested with *Sclerotium Rolfsii* should be planted to resistant varieties of peanuts in preference to attempting to rid the soil of this parasite by rotation.

(12) Observations during the seasons of 1915 and 1916 indicate that *S. Rolfsii* does not attack crimson clover planted in September or October for a cover crop.

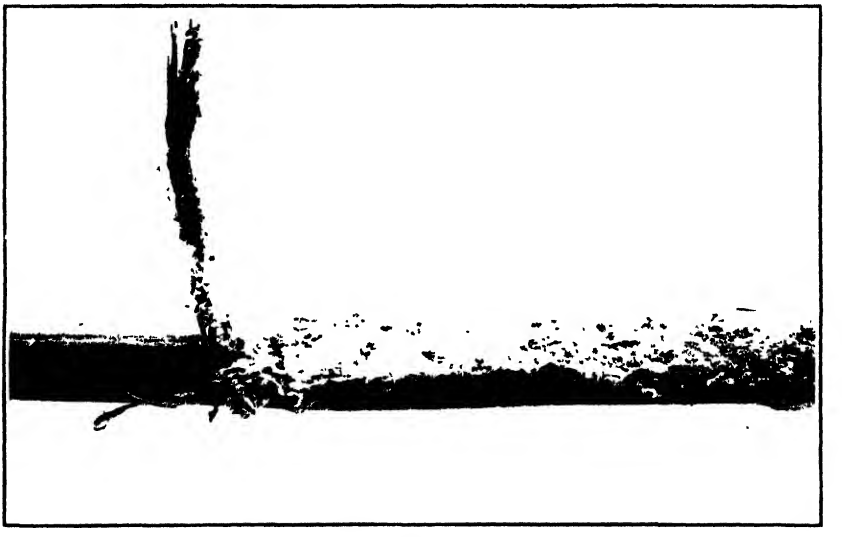
PLATE 96

A.—A hill of peanut plants completely wilted by *Sclerotium Rolfsii*. Original.

B.—A portion of a wilted shoot, showing the white mycelium and sclerotia of *S. Rolfsii* about its base. Original.

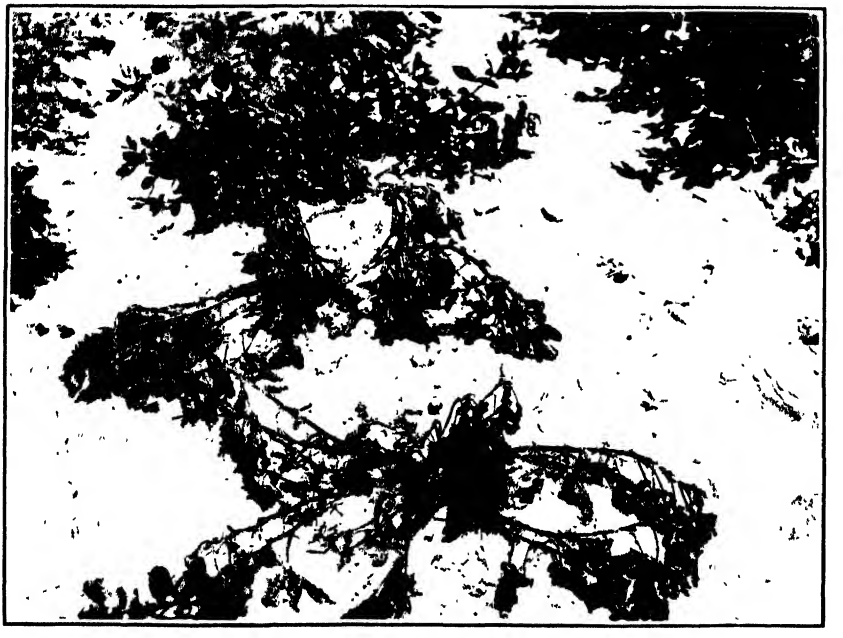


A



B

A



B

PLATE 97

A.—White mycelium and sclerotia of *Sclerotium Rolfsii* on fallen leaflets. Original.

B.—Three hills of peanuts which indicate that *S. Rolfsii* spreads from wilted plants and attacks adjoining healthy plants. Original.

TRANSFORMATION OF PSEUDOGLOBULIN INTO EUGLOBULIN

By W. N. Berg,
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INTRODUCTION

In several publications Banzhaf[2] states that when diphtheria serum is heated as it is in the preparation of antitoxin, part of the pseudoglobulin is transformed into euglobulin. Details regarding the methods of analysis or the analytic data on which Banzhaf based his conclusion were not found in the publications examined.

This transformation has both a practical and a theoretical interest. It facilitates the concentration of the antitoxin present in the serum by removing protein without removing any of the antitoxin, so that the final product contains all the antitoxin associated with much less protein. This is desirable because certain of the serum proteins have very little therapeutic value. In applying the heat treatment for the first time to a serum such as anthrax serum, it is obviously desirable to be certain that a similar transformation takes place. Otherwise, there would be no need of the heat treatment, and, besides, the heating may cause loss of potency.

On the theoretical side, the fact that pseudoglobulin can be transformed into euglobulin without affecting the total number of antitoxic units is almost conclusive proof that the antitoxin is a substance separate from pseudoglobulin. That this transformation may take place in some serums, but not in all, is indicated by the following experiments, in which the heating of the serum was carried out under carefully controlled conditions and the analytic data obtained by improved methods. In all, four horse serums were used, as follows:

Anthrax 48.—Serum obtained from horse 48; used in a previous work.[3] Blood drawn January 6, 1916. Potency high.

Anthrax 96.—Serum obtained from horse 96; used previously. Blood drawn January 6, 1916. Potency lower than serum 48.

Diphtheria 1.—A mixture of 500 c. c. of serum, potency 1,400 units per cubic centimeter when bled November 4, 1915, and 700 c. c. of serum, potency 250 units per cubic centimeter when bled October 29, 1915. From two horses.

Tetanus 1.—A mixture of 750 c. c. serum, potency 200 units per cubic centimeter when bled February 14, 1916, and 250 c. c. serum, potency 175 units when bled February 14, 1916. From two horses.

[1] The thanks of the writer are due to Dr. A. Eichhorn, Chief of the Pathological Division, for many valuable suggestions and his interest in the work.

[2] Banzhaf, E. J. The further separation of antitoxin from its associated proteins in horse serum. *In* Proc. Soc. Exp. Biol. and Med., v. 6, no. 1, p. 8–9. 1908.

—— The further separation of antitoxin from its associated proteins in horse serum. No. III. *In* Collect. Stud. Bur. Lab. Dept. Health, city of New York, v. 7, 1912–13, p. 114–116. [1913.]

—— and Famulener, L. W. The proteins and antitoxin in the serum of goats immunized against diphtheria. *In* Collect. Stud. Bur. Lab. Dept. Health, city of New York, v. 8, p. 208–212. [1915.]

[3] Eichhorn, Adolph, Berg, W. N., and Kelser, R. A. Immunity studies on anthrax serum. In Jour. Agr. Research v. 8, no. 2, p. 37–56, 1 fig. 1917.

When received at the Pathological Division these serums were in their native state, without preservative, and had not been filtered. Shortly after their receipt they were Berkefeld filtered and preserved with 0.5 per cent chloroform in a refrigerator.

With the analytical technic described in the previous publication by Eichhorn, Berg, and Kelser,[1] experiments were made on serums anthrax 48 and 96, diphtheria 1, and tetanus 1. The experiments on anthrax 48 and 96 were made in connection with the preparation of globulin for therapeutic use. After filtering the precipitated euglobulin, with or without heating, analyses were made of the filtrates. Obviously the more pseudoglobulin that is converted into euglobulin and precipitated, the less total protein should remain in the filtrate. A large number of analyses obtained on such filtrates, together with those obtained in the experiment in which diphtheria 1 and tetanus 1 were treated with ammonium sulphate, with and without heat, are omitted here because they were inconclusive. In analyses in which 10-c. c. portions of serum or filtrates corresponding to this amount are used, the unavoidable or unknown errors were great enough to obscure the effect of the heat treatment.

HEATING THE SERUM

In the following experiments the serums were heated in a water bath maintained at 61° C. The bath was heated by gas and was provided with a thermoregulator and an electrically driven stirrer. Numerous blank experiments (8) were made in which flasks containing water or one-third saturated ammonium-sulphate solution were heated. One standard thermometer was used for taking the temperature of the water in the bath, while two others were in the flasks being heated. The bath temperature varied from 61° C. by a few tenths of a degree, the bath having been so adjusted that during the experimental heating of the serum the temperature did not rise beyond 61.2° C. nor fall below 60.5° C. This drop was caused by the introduction of the flasks at room temperature, after which the bath temperature rose to almost 61° C. The bath contained about 15 liters of water.

The serum was heated in 200-c. c. Erlenmeyer flasks in quantities of 50 c. c. Two such flasks that were lightest and therefore thinnest were chosen from two dozen. These were always used for heating the serum mixtures. The object was to bring the content up to 60° C., hold it there for exactly 30 minutes, and then rapidly cool it. These two flasks were provided with perforated rubber stoppers, each carrying a thermometer graduated in whole degrees and tested by the Bureau of Standards. The thermometer used in the water bath was tested by the Physikalisch-Technische Reichsanstalt, and was graduated in tenths of a degree. Experiment A was on anthrax 48 and 96, both

[1] Eichhorn, Adolph, Berg, W. N., and Kelser, R. A. Op. cit.

being run at the same time. Experiment B was on diphtheria 1 and tetanus 1, both also run at the same time. On account of the breaking of a centrifuge tube containing a heated diphtheria mixture, the heating was repeated next day in duplicate, so that data were obtained on the heated and unheated single portions of anthrax 48, 96, and tetanus 1, and in duplicate portions of diphtheria 1.

The procedure with a single serum was as follows: Into each of two Erlenmeyer flasks of 200-c. c. capacity, 50-c. c. portions of the serum were pipetted. One of these flasks was always a thin flask used specially for heating. To each flask 25 c. c. of water and 32 c. c. of saturated ammonium-sulphate solution were added, making a total volume of 107 c. c. This procedure was then repeated with the second serum, as it was found convenient to run two together. The two flasks not to be heated were stoppered and set aside. The two to be heated were stoppered with stoppers carrying the thermometers. In the meantime the water bath had been in readiness at 61° C., and the two flasks were introduced. All three thermometers were carefully watched and the temperatures recorded at intervals not exceeding five minutes. The temperatures inside the flasks rose from room temperature, about 27° C., to 56° in the first five minutes of the heating, then to 59° in the next five minutes. After 10 minutes' heating the temperatures inside the flasks were exactly at 60° or below it by only a small fraction of a degree. The heating was then continued for exactly 30 minutes, during which time the temperature inside the flasks did not exceed 60°. It dropped a fraction of a degree for a few minutes on three occasions, but the bath temperature during this time was between 60.7° and 61°, and consequently for all practical purposes the serums may be regarded as having been heated for exactly 30 minutes at exactly 60°, with a preheating lasting 10 minutes. At the end of the heating period the flasks were transferred to a pan containing cold water. This brought the temperature down to that of the room in about five minutes. It is believed that this is as severe heating as is necessary in the preparation of globulin. In preparing large amounts of antitoxin, Banzhaf[1] brings the temperature of the mixtures up to just 60° which required two hours' heating in a water bath kept at 66°.

METHOD OF ANALYSIS

The next step is the separation and estimation of the precipitated euglobulin. In the following scheme of analysis the precipitations of euglobulin and pseudoglobulin are made under exactly the same conditions as in the antitoxin (pseudoglobulin) preparation, and consequently the analytic data may be applied to the corresponding globulin or antitoxin preparations without error. All four mixtures were transferred to 100-c. c. centrifuge tubes, which held about 110 c. c. when

[1] Banzhaf, E. J., 1913. Op. cit., p. 115.

filled to the top. After carefully draining the flasks the small amounts remaining in them were disregarded. The tubes were centrifuged for 25 minutes at about 2,500 revolutions per minute. The sedimentation was perfect and the euglobulin was firmly packed at the bottom of the tubes. The supernatant liquids were poured into 100-c. c. volumetric flasks. They may be poured through filter paper to make certain that no particles are poured off; but this is not necessary, as the euglobulin is sticky and firmly adheres to the bottom of the tube, which may be inverted without loss of precipitate. These 100-c. c. flasks should be weighed when dry and graduated in whole cubic centimeters on the neck, as the volume of liquid poured off may be more or less than 100 c. c. by 2 or 3 c. c. The volumes should be noted to be certain that they are the same for both the heated and unheated mixtures of the same serum.

The saturated ammonium-sulphate solution used was neutral to alizarin sulphonate, and it had been filtered through cotton and hard filter paper. The specific gravities of the saturated, one-half saturated, and one-third saturated aqueous solutions of ammonium sulphate were determined with a Westphal balance and found to be as follows at 26° C.: 1.250, 1.142, and 1.089. These figures were of value in calculating from the weight of the flask and contents the amount of supernatant liquid obtained. The supernatant liquid was used for the estimation of (1) pseudoglobulin, (2) albumin, and (3) these two together, in the form of total coagulable protein.

The precipitated euglobulin in the centrifuge tubes was dissolved in water and transferred to 400-c. c. beakers. These were heated up to the boiling point to coagulate the euglobulin, which separated out in large flocks. The addition of acid was not necessary, although in some instances 1 c. c. of *N*/5 acetic acid was added to favor flocculation. The precipitates were then filtered on weighed papers, washed free from sulphate, washed with small amounts of alcohol and ether, dried to constant weight in the air oven at 100° C., and weighed.[1] In only one instance was there any difficulty in securing flocculation—namely, in a heated diphtheria-euglobulin precipitate. When this occurs, there is, of course, a loss through the passage of unprecipitated protein into the filtrate, and generally the filtrate is very cloudy. That the result would be low in the case referred to (see Tables I and II) was noted before the determination was completed. The heated euglobulin apparently was different from the unheated. The latter dissolved readily in water, forming a clear solution, while the heated euglobulin dissolved much more slowly, forming a milky suspension which became almost water-clear on standing overnight.

Table I contains the results for euglobulin obtained from 50-c. c. portions of serum.

[1] For details see Eichhorn, Adolph, Berg, W. N., and Keiser, R. A. Op. cit.

TABLE I.—*Weights of euglobulin obtained from 50-c. c. portions of serum*

Serum.	Without heat.	With heat.	Increase in weight.
	Gm.	*Gm.*	*Gm.*
Anthrax 48	0.774	1.201	0.427
Anthrax 96	.547	.793	.246
Diphtheria 1	.406	.719	.313
Do	.435	[a].642	.207
Tetanus 1	.243	.304	.061

[a] Result low, probably due to incomplete flocculation.

The figures in Table I show unmistakably that more euglobulin precipitate was obtained from the heated serum than from the unheated. Obviously, it was desirable to ascertain definitely where the excess of euglobulin came from, and to check the correctness of these single results. Concordant duplicates do not prove correctness of results in these analyses; they do prove uniformity of technic and uniformity of error.

In the supernatant liquid (filtrate) poured off the euglobulin precipitates, the following determinations were made: Total coagulable protein (pseudoglobulin plus albumin), pseudoglobulin, and albumin. The method used was as follows:

TOTAL COAGULABLE PROTEIN.—Duplicate portions of the filtrate of 20 c. c. each, containing 9.35 c. c. of serum, were transferred to 400-c. c. beakers; about 300 c. c. of water were added and the mixture brought to a boil. The coagulum, consisting of pseudoglobulin and albumin, flocculated easily. This was filtered on weighed papers as usual, dried, and weighed. The results are tabulated as item C in Table II.

PSEUDOGLOBULIN.—A single portion of 25 c. c. of the filtrate, containing 11.68 c. c. of serum, was used. To this, 10 c. c. of saturated ammonium-sulphate solution were added, resulting in 50 per cent saturation. This was done in a 100-c. c. centrifuge tube and the mixture centrifuged for 20 to 25 minutes at 2,500 revolutions per minute. The pseudoglobulin packed firmly in the bottom of the tube. The supernatant liquid was poured off and its volume noted. This is the solution used for albumin determination. The precipitated pseudoglobulin is dissolved in water, transferred to a 400-c. c. beaker, heated, coagulated, filtered, dried, and weighed as usual. The results are tabulated as item D in Table II.

ALBUMIN.—Of the above fluid from pseudoglobulin precipitation 25 c. c., containing 8.34 c. c. of serum, was diluted and heated, and the coagulated albumin filtered, dried, and weighed as usual. The results are tabulated as item E in Table II.

The figures for euglobulin in Table II, item B, are the same as those in Table I divided by 5. No corrections were made for the volume of the precipitate in any case. The main objects of obtaining these data were, as stated before, to ascertain the source of the excess of euglobulin

in the heated precipitates, and to determine the accuracy of the method of analysis. Theoretically, A should equal the sum of B and C, and C should equal the sum of D and E. Although this equality is absent, the differences appear to be consistent, and indicate a uniformity of error due, possibly, to the absence of corrections for volume of precipitate, solubility of the precipitate in the wash water, occluded ammonium sulphate, etc. These errors, however, do not invalidate a comparison of the data in one column with those of the same serum in the next.

TABLE II.—*Analyses of heated and unheated serums.*

[All weights calculated to grams in 10 c. c. of serum.]

Serum.	Anthrax 48.		Anthrax 96.		Tetanus 1.		Diphtheria 1.			
	Unheated.	Heated.	Unheated.	Heated.	Unheated.	Heated.	Unheated.	Heated.	Unheated.	Heated.
	Gm.	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
A. Total coagulable protein	0.674	0.674	0.789	0.789	0.558	0.558	0.665	0.665	0.665	0.665
Do	.618	.618	.788	.788	.....	.....	.....	.....	.....	.....
B. Euglobulin	.155	.240	.109	.159	.049	.061	.081	.144	.087	[a].128
C. Pseudoglobulin and albumin estimated together	.437	.378	.668	.625	.447	.451	.534	.487	.523	.484
D. Pseudoglobulin	.290	.211	.568	.535	.385	.378	.475	.397	.479	.438
E. Albumin	.191	.191	.133	.135	.103	.103	.090	.098	.087	.097
Pseudoglobulin converted into euglobulin per cent.	27		6		0.2		16		9	

[a] Result low; see footnote to Table I.

DISCUSSION OF RESULTS

It will be noticed that the figures for albumin are practically identical in the heated and unheated serums. The figures for pseudoglobulin are consistently lower in the heated serums than in the unheated, which is an almost necessary consequence of the transformation of part of the pseudoglobulin into some protein having several of the precipitation characteristics of euglobulin. What is most important for the present investigation is that the loss of pseudoglobulin in the heated serums corresponds almost quantitatively with the gain in euglobulin in the same serums.

Obviously the figures for euglobulin, pseudoglobulin, and albumin are interdependent, and an error in one may cause a corresponding error in another. The figures for total coagulable protein, however, are independent of the others, and an error in them has no direct influence on the figures for the others. The two figures for total coagulable protein in the anthrax serums were obtained January 11, 1916 (upper figures), and May 9, 1916 (lower figures). The comparatively large difference between the two consecutive determinations in anthrax 48 may perhaps be due to the action of serum protease, which continued to digest the serum proteins. Chloroform does not prevent this action when the protease is active. The figures obtained were 0.676, 0.672, and 0.616

and 0.619 gm. The averages of these are the figures recorded in Table II. The corresponding figures for anthrax 96 were 0.756, 0.789, and 0.785 and 0.792. The first figure was rejected, being obviously erroneous. The corresponding figures for the diphtheria and tetanus serums were obtained February 28, 1916. The figures for euglobulin, pseudoglobulin, and albumin were obtained in May, 1916.

The percentage of pseudoglobulin transformed into euglobulin by the heat treatment is calculated by taking the difference between the pseudoglobulin in the unheated and heated serums and dividing by the amount of pseudoglobulin in the unheated serum. These results are tabulated at the bottom of Table II. Thus, for anthrax 48 the figures are $\frac{0.290-0.211}{0.290}$, which equals 27 per cent. However, this is not the only way to calculate this figure. The results for pseudoglobulin may be obtained by subtracting the figures for albumin from those for pseudoglobulin plus albumin. If the percentage of transformation be calculated from the lower values for pseudoglobulin, the figures are 24, 8.4, − 1.2, 12.4, and 11.2, respectively, reading across the bottom of Table II. It is expected that further work on the methods of analysis will yield better results.

From Table I it is apparent that the increase in euglobulin is easily determined when a 50-c. c. portion of serum is used. When, however, the filtrate is divided into several portions for the other determinations, the errors in analysis become proportionately large. It is furthermore apparent that in general the amount of pseudoglobulin transformed is not large, considering the long period of heating at a comparatively high temperature—that is, 30 minutes at 60° C. The extremely small amount of the transformation in the tetanus serum leads to the inference that while the transformation may take place when serums are heated, it does not necessarily always take place. This might not be noticed when handling large volumes of mixed serums.

This work was completed several months before the recent investigations of Homer[1] came to the author's attention. Homer states (p. 291) that there is no conversion of pseudoglobulin into euglobulin when serum mixtures were heated to 61° or 63° C. It is probable that the failure to observe the transformation was due to the use of analytical technic that was not delicate enough and to the errors incidental to the handling of large amounts of serum mixtures, as in the routine preparation of antitoxin. Better analytical results can undoubtedly be obtained when using small amounts of serum under conditions adapted to exact analysis and separate from antitoxin preparation.

[1] Homer, Annie. On the concentration of antitoxic sera. *In* Biochem. Jour., v. 10, no. 2, p. 280–307. 1916.

—— An improved method for the concentration of antitoxic sera. *In* Jour. Hyg., v. 15, no. 3, p. 388–400. 1916.

CONCLUSION

The transformation of pseudoglobulin into euglobulin was observed in four serums that had been heated 30 minutes at 60° C. in the presence of 30 per cent saturation ammonium sulphate. In some instances the amounts transformed were considerable, although in one of the serums the amount was so small as to indicate that the transformation does not take place in all serums.

The methods of analysis were improved by the use of the centrifuge as a means of separating globulin precipitates from their filtrates. The precipitations in the analyses were made at the same dilutions as in the precipitations of globulin for therapeutic use.

DISSEMINATION OF THE ANGULAR LEAFSPOT OF COTTON

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INTRODUCTION

A satisfactory explanation of the dissemination of many of the diseases of plants is lacking. The literature recognizes such agents as insects, wind, tools, laborers, drainage, spattering rains, etc., and yet in some cases one must doubt the importance attached to these by investigators. In other instances, particularly in the cases of bacterial diseases affecting the leaves, stems, flowers, and fruits, no satisfactory explanation has been offered. This was the status of affairs in the case of the angular leafspot of cotton (*Gossypium* spp.) when the author began the investigation of this disease at the South Carolina Experiment Station. It is the purpose of this paper to present the data obtained during the past summer and to offer the conclusion reached as to its dissemination under the conditions existing in western South Carolina, with a suggestion of the possible importance of these factors in the dissemination of other similar diseases.

HISTORICAL RÉSUMÉ

A brief résumé of the literature dealing with some of the most common bacterial diseases follows, being presented in order that the true situation may be understood and that the information found there may be used in this discussion.

Beach (1893)[2] and Halsted (1893) concluded that the blight of beans was a bacterial disease, because of the constant association of bacteria with the typical lesions. Halsted further stated that the disease was carried over in the seed. Sackett (1909, p. 21) states that—

> Rain and dew are doubtless agents in spreading the germs from one part of the plant to another by washing them from old lesions onto unaffected parts,

though no evidence of this fact is given. Edgerton and Moreland (1913) successfully inoculated plants without wounding, and concluded that infection can take place through the stomata.

Pierce (1901) successfully inoculated the fruit of the walnut with water suspensions of *Bacterium juglandis*. R. E. and C. O. Smith and

[1] The author is greatly indebted to Prof. R. A. Harper, of Columbia University, for the perusal and criticism of the manuscript, and to Mr. J. W. Sanders, assistant in this laboratory, for his most careful and untiring aid in the work.

[2] Bibliographic references in parenthesis refer to "Literature cited," pp. 473-475.

Ramsey (1912) agree that the uninjured host is susceptible, and conclude that water is apparently the principal agent in conveying the bacteria from the existing lesions to younger leaves and small nuts lower down on the tree. They state (p. 338) that—

> During one of these [before mentioned] fogs the trees became saturated, water dripping from one portion of the tree to another which could easily carry the disease organisms to healthy tissue. Observations go to show that secondary infection in which large numbers of the small nuts become diseased is very likely to follow one of these foggy periods.

Arthur and Bolley (1896) suggest that wound-producing insects are probably an important factor in the spread of the carnation disease caused by *Bacterium dianthi*, though infections can also take place through the stomata. They recommend a method of watering the plants in which the foliage is kept dry, and experience has shown the efficacy of it. In their conclusion (p. 34) the authors state that—

> As there must be moisture upon the leaves sufficient to enable the bacteria to move about and enter the stomata in order that they may gain access to the interior of the leaf, it is evident that keeping the foliage dry will prevent the disease.

It apparently does not occur to them that the water under pressure, being dashed from plant to plant, might serve as a means of dissemination.

Lewis (1914) describes a disease of *Erodium* spp. and *Pelargonium* spp. caused by *Bacterium erodii*, which, he says (p. 230),—

> is more prevalent in crowded beds where the plants remain moist and light is not so dense.

Sprinkling is suggested as the most probable method of dissemination.

Sackett (1910) discusses a disease of the stems of alfalfa caused by *Pseudomonas medicaginis*. The organism is probably carried from place to place on wind-blown dust particles. O'Gara (1914) describes more fully the characteristic appearance of the affected parts, and adds that stomatal infections may occur, though by far the greatest infection takes place through openings produced by insect puncture and severe frost injury.

E. F. Smith, in the second volume of his work on Bacteria in relation to plant disease (1911), summarizes the data relating to water-pore inoculation of cruciferous plants with *Bacterium campestris*, and, in discussing Fischer's objections to this conclusion, writes (p. 308)—

> * * * (2) the hypothetical, dust-dry, wind-borne bacterium requiring a half day or more to moisten it, is probably not the one that usually enters the water-pores and induces the disease, but rather a fresh germ recently come from the interior of some affected leaf as an extrusion from some water-pore already diseased, or left in the vicinity of the water-pore by some wandering insect. * * * such a bacterium would be ready to grow as soon as it found lodgment in a moist place.

The disease progresses most in periods of frequent rains. Russell (1898, p. 31–32), writing on the same subject, states:

> One direct agent by which the disease is spread is the wind. Whether the disease germ is present in the soil or in decaying plants, the dried bacilli can be carried through

the air * * * Inasmuch as this [infection through the water pores] is by far the most common gate of entrance for the disease organism, it is highly probable that the disease is disseminated by means of the wind more than in any other way.

Smith discusses the wilt of cucurbits in the same volume and presents evidence of the dissemination of that disease by insects, especially one *Diabrotica vittata*. Concerning Cobb's disease of sugar cane, this author in his third volume (1914) writes (p. 48):

We can well imagine, however, that under ordinary field conditions, with an abundance of dew or rainfall, and plenty of insect depredators, diseased plants might readily infect neighboring healthy ones, especially when young.

In the chapter on Stewart's disease of sweet corn, which is also in the third volume, the author states (p. 124), in the discussion of an experiment relating to seed infection:

If the disease was actually derived from the seed-corn there probably would have been some cases during the seedling stage, and fragments of these soft plants full of the bacteria would have been blown upon neighboring plants, or dragged by cultivators, or carried on the feet of men and horses, or bitten into by insects, or washed about by rains and dews. There are ways enough to account for the dissemination of the bacteria in the infection of a few plants when the distance is only a matter of a few feet.

In an address before the Massachusetts Horticultural Society (1897) Smith discussed the subject under the headings: (1) Spread by insects; (2) spread by snails and slugs; (3) spread through manure pile; (4) spread by way of the soil; (5) spread by way of seeds, seedlings, buds, tubers, cuttings, and nursery stock.

Macchiati (1891) and Boyer and Lambert (1893) describe a leaf and twig disease of mulberry caused by bacteria. Both claim to have isolated the organism, and the latter authors report successful inoculations with a bacterium named by them "*Bacterium mori.*" E. F. Smith (1910) uses this name[1] for an organism which he determined to be the cause of the same disease in Georgia, though it differed from that of Boyer and Lambert. In no case, however, is an explanation of the method of dissemination offered.

Manns (1909) concluded that the bladeblight of oats was caused by two bacteria to which he assigned the names "*Pseudomonas avenae*" and "*Bacillus avenae*," and that these bacteria were present in the soil reaching the host through "spattering rains." Manns and Taubenhaus (1913) report their studies of the streak disease of sweet peas and clovers which they found caused by a bacterium named by them "*Bacillus lathyri.*" Later, Manns (1915) discusses this disease and considers more fully the subject of dissemination, saying (p. 12) that the disease attacks the plants about the beginning of the blooming period—

having its origin usually near the ground, indicating distribution by spattering rain and infections through the stomata.

Neither of them states by what means the upper parts of the plants become infected.

[1] According to Migula's system of classification this name would be "*Pseudomonas mori*"

Griffin (1911), Barss (1913, 1915), and Rees (1915), writing separately of the gummosis of cherry trees, agree that the disease is caused by a motile bacterium, and suggest that sucking insects are the probable agent in its dissemination. Barss (1915, p. 239) says:

> The exact manner in which the disease is disseminated is not known, but indications seem to point to the possibility that sucking insects may be largely responsible for infections.

No data are available in the literature as to the susceptibility of any part of the plant without previous injury, all inoculations having been made by needle puncture.

Stewart and Leonard (1913) show clearly that certain sucking insects, among them the tarnished plant bug, are capable of inoculating tender shoots of pears when *Bacillus amylovorus* is present at the immediate point of puncture or upon the mouth parts of the insect. These authors (1915) conclude that certain flies are not active agents of inoculation, but, because of their abundant presence in the orchards and among nursery stock, they are probably a factor in spreading the bacteria from place to place. Attention is attracted by Stewart (1913, 1915) to the increased disease following rainy weather, due to both the increased susceptibility of the host because of the more succulent growth and the increased activity of the bacteria because of more favorable environment. Heald (1915) is of the opinion that leaf infection of pears by fireblight is possible through water pores, while Hotson (1915, 1916) offers further evidence in support of this.

Rolfs (1915), writing on the disease of stone fruits caused by *Bacterium pruni*, states that infection takes place through stomata and that a film of moisture is necessary to successful inoculation. He says (p. 416, 421):

> Rain and dew are not only important factors for inoculation, but they also carry the bacteria to the healthy leaves, twigs, and fruits, and thus frequently serve as agents of transportation. * * * The warm, slow, continued rains of the summer furnish the best conditions for the rapid spread of the disease. Heavy, driving rains of short duration followed by sunshine and winds are not favorable to its spread, since many of the bacteria are washed to the ground, and the leaves are quickly dried off and the few bacteria that may be spread will be quickly dried and killed.

Rolfs (1915), in his discussion of the angular leafspot of cotton, states (p. 17–18):

> Wet weather, of course, materially aids in the dissemination of the organism. Even if the weather is excessively dry, the dew at night will often furnish sufficient moisture for inoculation. * * * If for any reason the first leaves fail to become inoculated in this way [by contact in presence of dew] the movement of the leaves in the wind especially during a storm, will soon bring them in contact with some of the virus on diseased leaves. * * * The rain carries large numbers of the organism to the new tissue and to the ground under the plants. * * * The soil under the infected plants may thus become an important means of inoculating many of the lower leaves.

Hasse's (1915) conclusion that *Pseudomonas citri* is the cause of Citrus canker has been confirmed by Wolf (1916). The latter states (p. 94), regarding its dissemination:

Definite experimental data are wanting on the agencies by which Citrus canker is spread * * *. It is evident that rain and dew are important factors in carrying the disease to unaffected leaves, twigs, and fruits of trees in which the disease is already present.

Even though Stevens (1914) thought the disease to be due to a fungus, he writes (p. 41):

The disease seems to develop and spread rapidly during rainy weather, but it is more or less retarded during periods of drought or in a dry season.

Brown and Jamieson (1913) present their work on a disease of nasturtium and sugar-beet leaves caused by the same organism, which they named "*Bacterium aptatum.*" While the authors state they had no opportunity to study this disease under field conditions, their experiments would suggest that infection takes place only in bruised or wounded tissue caused by insects or mechanical injury.

A number of preliminary reports of work on bacterial diseases have been made in recent years; and, since in most of these the subject of dissemination is not mentioned, the present author takes it that this phase will be discussed later and, therefore, he will not review this literature.

The facts in the dissemination of certain types of fungus diseases have a close bearing on the subject; yet this literature is so voluminous as to be impossible of review here. However, because of its relation to one of the most serious cotton diseases, anthracnose, Whetzel's (1906) paper on bean diseases is mentioned. Of anthracnose, the author writes (p. 205):

The spores may be scattered by the cultivator, the pickers, by animals, or by the wind in damp or rainy weather.

EXPERIMENTAL INVESTIGATIONS

Infection of the leaves of cotton by *Bacterium malvacearum* can easily be brought about by superficial inoculation in the presence of sufficient moisture. This has been done by applying small amounts of agar-slant cultures of the organism with glass rods and spreading with rubber-gloved fingers when the dew was on the leaves, by applying water suspensions of the bacteria with cotton swabs at all times of the day or evening, spraying such suspensions with an atomizer, or by painting them upon the leaves with camel's-hair brushes. The first signs of infection are minute, dark-green, angular (triangular or quadrilateral) spots on the underside, whether the inoculation was made upon the upper or lower surface of the leaf, usually in 7 to 10 days, though often not earlier than 15 days. In a day or two following the first appearance on the underside of the leaf the same dark-green water-soaked spot will appear on the upper side, though less conspicuously angular. When held up to the light, such spots show a translucency as contrasted with the light, impervious normal leaf and the irregular transparency of some insect injuries. The spot usually increases in size simultaneously on both sides of the leaf, but never crosses the veins. Single infections seldom increase to a size larger than 3 to 4 mm. in the longest dimension. The larger spots, so con-

spicuous on the leaves, are usually caused by the coalescence of two or more separate infections which often occur in clusters when in the mesophyll regions and in a linear direction when along the veins, seemingly where the water gathers and is last to evaporate.

The top side of the spot thus appearing becomes a reddish brown color over a circular area at the center and shrinks slightly in thickness. The colored area increases in size until it conforms to the angular shape of the affected part, the red advancing to the margin and the center shading off to gray. On the underside the spot becomes a brown color, though the dark green, characteristic of the young spot, remains as a narrow band about the margin until the latest stages of development, a place where the activity of the disease continues longest. When the green band finally disappears, the brown replaces it, giving a sharp angular margin to the spot. These color changes may develop quickly, so that the entire spot will be brown eight or nine days after inoculation; but usually they proceed more slowly, leaving the spot dark green for five or six days.

SEED INFECTION

The source of bacteria for the first infections of each season is as yet undetermined, because of conflicting experimental results obtained during the past summer; however, such a problem can be solved satisfactorily only when great numbers are used under the most favorable circumstances. One experiment, or series of experiments, can not be expected to settle the matter.

Over 2,500 seedlings from seed of various sources grown during the winter in all variations of temperature and moisture in the greenhouse and laboratory failed entirely to show the disease. Two instances of cotyledonary infection occurred in the late spring upon seedlings in the greenhouse. An acre field planted on April 20 was entirely free from the disease until August, except in such portion as had been used for inoculation experiments. Another field near by, planted partly with these same seeds and partly with others on May 15, developed considerable disease in both lots of seedlings, though the season was rainy and the chopping was delayed; and no data as to the amount of disease due to the seed and that due to local dissemination could be secured. It was true that, of the diseased seedlings examined, equally as many had cotyledons free from the disease (84) as had them spotted (78).

In order to decide more carefully the probability of seed infection, an experiment was arranged in which samples of various lots of seeds were planted in plots, seven rows wide, the rows being 50 feet long and 3 feet apart. Because of the triangular shape of the land available, these dimensions could not be strictly adhered to; yet each lot of seed was represented by one such plot at least. Most seeds used were samples obtained from several growers in various parts of the State, who had fields badly infected early in the summer, such as would likely be due to

a high percentage of seed infection. These are labeled "Lots II–V," Lot I being seed of the same lot planted on April 20 and remaining free from seedling infection. The other lots (VI–X) are Mississippi Cook seed grown on the Experiment Station farm under differing conditions the previous summer.

On two sides of this triangular plot were cotton variety tests of the Station, and the disease was present upon these plants. To guard against insect activity in spreading the disease to the young seedlings and thus confusing the results, cages of cheesecloth were erected over 12 feet of a row of each lot of seed, giving an opportunity for the disease to appear under such protection if the bacteria were present upon or in the seed. The field was planted on August 12. The seed germinated quickly, and the seedlings began to appear above the ground by the 16th and 17th. On the 22d a careful examination was made of all the plants, but no disease was found. On the 28th 11 cases of cotyledonary infection were found, and on September 1, 20 days after planting and 15 days after the cotyledons were spread, affording ample time for the appearance of the disease in view of our earlier work, the final counts were made. Of 34 diseased plants found, only one was beneath a cage; yet that one lends weight to the conclusion that seed infection does occur. The tabulated data by plots of each lot of seed follows in Table I.

TABLE I —*Results of the infection of cottonseed by angular leafspot*

Lot No	Plot No	Number of seedlings.	Number diseased in —		Total number of seedlings.	Total number diseased	Percentage diseased
			11 days.	20 days			
I	1	820	0	0			
	2	4,926	0	1			
	3	1,743	0	0			
	4	586	0	0			
	5	468	0	0			
	Cage.	207		0	8,750	1	0.011
II	6	3,023	4	2			
	7	3,543	0	7			
	Cage.	179		1	6,745	14	.2
III	8	2,622	4	1			
	9	700	0	0			
	Cage.	86		0	3,408	5	.14
IV	10	2,884	0	6			
	11	1,242	0	4			
	12	1,402	0	8			
	Cage.	117		0	5,645	18	.31
V	13	142	0	0	142		
VI	14	2,030	0	0	2,030		
VII	15	1,150	3	4			
	16	195	0	0			
	Cage.	145		0	1,490	7	.46
VIII	17	2,002	0	0			
	18	1,247	0	0	3,249	0	
IX	19	3,678	0	0	3,678	0	
X	20	1,415	0	0			
	Cage.	16		0	1,431	0	
Grand total					36,568	45	

In this experiment the seedlings of Lot I were slightly diseased (0.011 per cent), the results being comparable with those obtained from the field planted on April 20. It is improbable that the diseased plants in the field planted with these seed on May 15, which varied from 2.7 to 8.1 per cent of the plants of single rows, were due to seed infection, but rather to the spread of the disease from other infected plants at an extremely favorable time.

The other results obtained, 0.2, 0.14, and 0.31 per cent, do not account for the amount of disease observed in fields planted with these seed. The field planted with seed of Lot V was the most badly diseased of any seen on a trip about the State in June; and, while the author obtained only 142 seedlings, none of them were diseased.

Without doubt, 0.46 per cent of diseased seedlings at the beginning of a season, especially if rainy weather prevails before chopping, would be sufficient to start a general field infection, and this could be called the primary infection. It is interesting to note in this connection, however, that 1,218 seedlings grown in the greenhouse from seeds planted on August 5 and taken from the same bag as those in Lot VII were entirely free from the disease as late as August 29. It is improbable that the conditions in the greenhouse were unfavorable to the development of the disease, since successful artificial inoculations have been made here besides the two cases of natural cotyledonary infection already mentioned; and, if we accept this as the case, adding these seedlings to those observed in the field, making a total of 7 diseased seedlings in 2,708, the 0.46 per cent is reduced to 0.25 per cent.

Whatever the true situation in this regard may be, and only further carefully checked observations can decide, it is a fact that the disease appears sooner in some parts and later in others, yet almost inevitably in every cotton field. In any method of primary infection it is improbable that every plant will be attacked, so that the spread of this disease from leaf to leaf and plant to plant becomes a subject of considerable interest and importance.

INSECT DISSEMINATION

In view of all the work—much of it recent—done on the subject of the spread of plant diseases by insects, an effort was made to determine carefully the extent of their activities in the dissemination of the angular leafspot. Leaf-eating beetles (flea beetles and cucumber beetles) were abundant in some fields of seedling cotton, but in only one case has the author observed the disease developing about the margin of eaten areas. The most common insects upon the older plants were the jassids; and, since they were more active than any other except the ants (the latter, however, being active only after the dew had disappeared), especial attention was given them. Five large plants were

caged in the greenhouse, with cheesecloth stretched over wooden frames. Into one of these cages were placed 50 jassids caught in the early morning while the dew was on the plants; into another, 75 jassids caught about 9 a. m., after the dew had evaporated. Two cages were used as uninoculated controls, while the plant in the fifth cage was sprayed with a water suspension of a 6-day-old 1 per cent saccharose agar slant culture of *Bacterium malvacearum*. After the insects had been upon the plants for 18 days in one instance and 14 days in the other and after the inoculated control had developed for 11 days the cages were opened and observations made. The results are given in Table II.

TABLE II.—*Results of a greenhouse experiment to determine the agency of insects in the dissemination of angular leafspot*

Test.	Number of leaves not infected.	Number of leaves infected
Jassids caught in dew (18 days)	58	1 (1 spot?).
Control	58	0
Jassids caught dry (14 days)	132	0
Control	124	0
Inoculated control	99	22 (871 spots).

While the data are not conclusive as to the activities of these insects the conclusion that they have a very slight effect, if any at all, is supported by the later developments in the same patch used for seed-infection studies and described above.

Shortly after September 1 this field was chopped to a close stand and the plants allowed to develop. There was considerable angular leafspot present upon the plants in the adjoining fields, and an excellent opportunity was presented for the insects to demonstrate their influence upon the spread of the disease, since the growing plants were at a favorably susceptible age. A careful examination was made of this field on October 2, particularly of those plots lying next to the older cotton. Table III shows the amount of disease present, the data being presented in rows per plot. Those of the first five plots were parallel to the rows of the adjacent cotton field, the seventh row being slightly over 21 feet away, while the remaining plots were bordering the cotton on another side of the triangle, with the rows running toward the other field, so that no idea of distance can be had. The numbers of the plots are identical with those in Table II, which facilitates comparison.

TABLE III.—*Results of a field experiment to determine the agency of insects in the dissemination of angular leafspot*

Row.	Plot 1.		Plot 6.		Plot 14.		Plot 8.		Plot 2.		Plot 10.		Plot 9.		Plot 4.		Plot 5.		Plot 12.		Plot 18.	
	Plants.	Plants diseased.	Plants.	Plants diseased.	Plants.	Plants diseased.	Plants.	Plants diseased.	Plants.	Plants diseased.	Plants.	Plants diseased.	Plants.	Plants diseased.	Plants.	Plants diseased.	Plants.	Plants diseased.	Plants.	Plants diseased.	Plants	Plants diseased.
1	41	1	49	0	35	0	46	1	80	0	51	4	25	0	19	0	69	0	52	1	46	0
2	35	0	50	0	41	0	38	1	78	0	54	2	26	0	21	0	64	0	47	0	31	0
3	31	0	53	0	40	0	56	0	83	0	45	1	20	0	20	0	.....	...	41	0	32	0
4	21	0	48	3	38	0	49	0	75	0	48	0	23	0	16	0	.....	...	37	0	29	0
5	21	0	61	0	37	0	53	0	65	0	42	0	18	0	14	0	.....	...	.....	..	17	0
6	9	0	65	2	44	0	43	0	58	0	60	0	19	0	16	0	.....	...	.....	..	15	0
7	6	1	52	0	44	2	44	0	47	0	36	2	0	0	11	0	.....	...	.....	..	.....	...
Total	154	2	378	5	279	2	329	2	486	0	336	9	131	0	97	0	133	0	177	1	170	0

Considering the irregular distribution of the diseased plants in plots 1, 6, 14, 8, and 2, little evidence of insect activity is noted; and this fact, together with the higher percentage of diseased plants in plot 9, which was credited with high seedling infection (Table I), might lead one to believe this disease present to be more likely due to seed infection or spread from such plants.

DISSEMINATION BY WIND DURING RAINFALL

An extensive inoculation experiment was arranged in one of the college cotton fields to demonstrate beyond all doubt the pathogenicity of the organism which preliminary inoculations indicated was *Bacterium malvacearum*. This field was located on a plateau overlooking a valley 2 miles wide sloping from east to west, with no higher hills near by. The row chosen for the experiment was parallel to a roadway and about 20 rows from it (80 feet). The inoculations were made on May 26, one person applying the bacteria from agar-slant cultures to the underside of two or three leaves of each plant by means of a sterile glass rod. One tube culture and one rod were used as far as the culture served, when another culture and rod were used. The bacteria were gently spread over the underside of the leaf with the fingers by a second person, using rubber gloves to facilitate sterilization after each culture. The success of the experiment was noted on June 3 (eight days later), though a complete record was not taken until June 5, when an examination showed that none of the control plants were diseased and that all those inoculated were diseased except two. These also showed infection shortly afterwards.

A record was taken on June 5 of the number of leaves infected and uninfected. Comparison with a similar record taken on June 15 showed an increase from 2 to 10 leaves per plant, usually 3 to 6, and a similar

increase of diseased leaves. At the time two control plants standing next to inoculated plants were also diseased. Thus, there is without doubt a rapid increase of the number of the spots on infected plants and those adjacent, probably due, as Rolfs (1915, a) points out, to the spread of the bacteria by contact with uninfected leaves and plants in the presence of sufficient moisture.

During the observations made on June 26, to note the spread of the disease in this field, it was found that the row on each side of that inoculated contained infected plants, though the disease appeared to be spread farther toward the east than the west. That part of the field was carefully diagramed, an accurate record made of the disease on each plant, and the data then charted for closer study. The chart is reproduced here to show the true situation in the field at that time (fig. 1, *B*.)

All the plants are represented by cross marks, those originally inoculated with *Bacterium malvacearum* are divided into series, each series being given the number of our culture used. The numbers near the plants of the neighboring rows and of the checks designate the number of angular leafspots found on them on June 27 and 28. The inoculated plants were so badly diseased as to make such data useless.

Two facts stand out upon examination of the chart: (1) There had been very little spread of the disease in a westerly direction as contrasted with that toward the east, and (2) the spread toward the east was strikingly opposite the inoculated plants as compared to the control plants. Upon closer study of these points it will be noticed that one had to proceed 14 rows to the east to find a row comparable in amount of disease with the second one west of the inoculated row. Further, a decrease in disease is noticed west of wide skips in the inoculated row almost as pronounced as that west of the control plants.

The significance of these data was much of a surprise to the author; therefore, he immediately set about to duplicate the original conditions, hoping at least to obtain some partial repetition of these results. A row in the same field, 35 rows west of the first inoculated row, was chosen for the second experiment. For this inoculation a bacterial suspension was made by grinding a large number of badly spotted leaves in a food chopper and diluting the macerated tissue with water. The rapid spread of the disease after the first inoculation led the author to believe that the bacteria in culture had been more or less attenuated, since it was eight days after inoculation before the disease appeared on the leaves. Unless a great amount of this spread had taken place at one time, it would have been difficult to understand how the disease could progress so far in one direction if each spot took 8 to 10 days to appear.

In the row used, a number of plants serving as controls were not inoculated, a number were inoculated, and others again left as controls. A shallow dish of the fresh suspension of bacteria was held in one hand while each of the leaves of the plants to be inoculated was immersed;

in this way inoculations were made with bacteria which had never been in culture. A record made of the amount of disease in this row at the time of inoculation is shown in figure 1, *c*. There were some infected plants, but not enough to interfere with the work as long as their location was known. Frequent examinations of the plants were made; and any idea of attenuation of the virility of the organism by artificial culturing proved unfounded, since few of the spots appeared in less than eight days and most of them later. A record taken on July 15 (fig. 1, *c*) shows how slight the inoculation was, probably because of the high dilution of the bacterial suspension. No count was made, and no idea is now had of the number of bacteria per cubic centimeter of that suspension.

It was fortunate, from the author's viewpoint, that this experiment was arranged before the prolonged rainy period of July, in which rain fell on July 7 and almost every day afterwards until the 25th. During this time the effect of the two tropical hurricanes, which did considerable damage in the Gulf States, was felt, the wind blowing during all the time from the southeast.

Observations made toward the end of July, allowing a time factor as long as we had in our first experiment, showed that the disease had spread to the northwest of the inoculated row with very little disease on the row to the east (fig. 1, c). Upon further examination of the plants in the first experiment the disease was found to have spread over the plants in the rows west of that inoculated (fig. 1, *A*), the direction of spread having been reversed. Many of these plants were recorded as being free from disease on June 28, while on July 30 it was so abundant as to make careful counting needless. Consequently "1,000" is used to denote relatively a great amount of disease, more than 1,000 spots. The only obvious factor which is capable of such far-reaching action is the wind, and this only when sufficient moisture is present over the leaves to enable the bacteria to become detached from the colony within the diseased area, either through the stomata or from the surface of the spots. Even heavy dews afford sufficient moisture for this purpose, as has been demonstrated by experiment.

Absorbent cotton, sterilized in a plugged Erlenmeyer flask, was carried to the field on two occasions in the early morning while the dew was present. Small bits of this were pulled off with forceps, and each bit placed upon the top side of the diseased leaves, not especially over lesions, but promiscuously upon the leaf. After these were placed, each was taken up and placed into a tube containing sterile water (a sterile water "blank"). These were taken to the laboratory, shaken thoroughly, and samples of the water plated in agar. In the first preliminary experiment 5 out of 30 plates showed *Bacterium malvacearum*. In the second case, 12 out of 84 plates showed the organism. It would seem that the close percentages (16 and 14) of these two experiments is merely coincidental;

yet in another experiment in which cold, poured agar plates were taken to the field and with covers removed, dew dashed onto the agar surface, about the same percentage gave growths of *Bact. malvacearum.*

It is reasonable to expect that the bacteria would become as free in a film of water due to rains as to dew. When bacteria have thus escaped from the interior of diseased areas through stomata (possibly in other ways) and have been suspended in this water film, any agency carrying this water from plant to plant becomes a means of dissemination. Wind during rainfall is the most probable agency which has been active in this disease of cotton. Infection by contact of plants is precluded by the distance between rows (4 feet) and the height of the plants at the time of the first experiment—8 to 10 inches at its beginning and not over 18 inches high at its close.

METEOROLOGICAL CONDITIONS

Little can be said concerning the wind during the rainfall which spread the disease in these experiments. Future studies are planned in which these factors will be more closely observed, and it is hoped to learn what variations and what minimum amounts of wind and rain, separately and together, will have this effect. It is a fact of record that the wind blows during the rainfall of thunderstorms of this region, and it is common knowledge that the wind blows violently during the rainfall of West Indian hurricanes. As types of the thunderstorms most important in this regard, three graphs are presented (fig. 2), records of storms observed at the office of the United States Weather Bureau, Little Rock, Ark., and Vicksburg, Miss. The high wind preceding the rain, or at its beginning, should be noted. It is improbable that this wind at the beginning is instrumental in spreading bacterial diseases, but it will be noted that later the wind reaches a velocity of 25, 29, and 35 miles per hour during periods in the storms when rain is falling heavily and after the foliage has been wet for some time. It is extremely probable that winds of these velocities blowing during heavy rainfall will serve to disseminate such diseases as the angular leafspot of cotton.

The records of storms in the extreme western portion of the cotton belt are used here so that it will be noted that these meteorological conditions are not peculiar to this locality but that such conditions are prevalent throughout the cotton-growing States and probably elsewhere as well.[1]

DISCUSSION OF AGENCY

Suitable conditions of wind and sufficient moisture occur during the summer rains of this region. The method of action of this combination has been and will be the subject of close attention. During June, 1916,

[1] Thanks are due to Messrs. H. S. Cole and William E. Barron, officers in charge of the Weather Bureau offices at Little Rock and Vicksburg, respectively, for their kind assistance and suggestions in this phase of the subject.

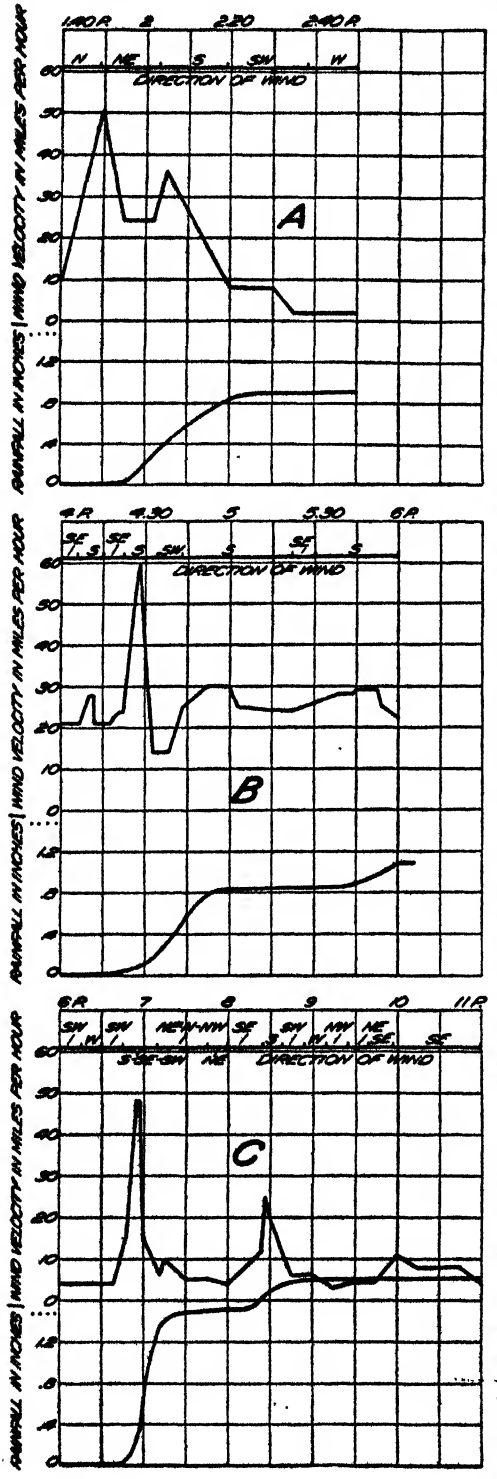

FIG. 2.—Graphs showing direction of wind and its velocity and the amount of rainfall during thunderstorms: *A*, Little Rock, Ark., August 22, 1916; *B*, Little Rock, Ark., June 5, 1916; *C*, Vicksburg, Miss., September 25, 1916. Note the high wind velocity during the rainfall in the latter part of the storms.

there occurred one or two typical thunderstorms. During July a rainy period continued through the greater part of the month, though from figure 1 it can be seen that the inoculated plants in the second experiment were only slightly diseased on July 15, most of the spread probably occurring after that time. During this time, however, the effects of two West Indian hurricanes were felt, and the wind blew during most of the rain.

Just how far a falling raindrop could splash water from the surface film of a leaf is hard to conjecture, but this must be a factor in the problem. It is probably this that previous authors had in mind in discussing disease dissemination; yet the distance which the wind blowing at the time of the splash will carry this ascending water is a problem for determination. The possibilities of this chain of action during a driving rain are considerable if one includes the distance bacteria may be carried from the original lesion, then splashed up again and carried farther, and so on, until a dilution too great for infection is obtained.

Rolfs concluded that rains followed by sunshine and wind were not favorable to the spread of the bac-

terial disease of peaches, etc., since the desiccation resulting killed the bacteria which had been spread. The physiology involved in our subject has not as yet been determined—whether negative heliotropism, positive chemotropism, or mere chance determines the time within which the bacteria enter the stomata—but it is true that infection resulted almost uniformly when the inoculation was made in direct sunshine on the upper or lower surface of the leaves with a temporary unprotected layer of water over the leaf surface. It may have been that a small percentage of these organisms withstood the unfavorable environment until night and that penetration of the leaf occurred then. The result of the author's experiments on the effect of sunlight upon *Bacterium malvacearum* agree with those of Rolfs (1915, a) and Edgerton (1912), in that the present writer found that 15 minutes' exposure kills some bacteria, 30 minutes a considerable number, and in an hour almost total destruction of the bacteria in the exposed parts of poured agar plates occurs. Some growth always appeared at the edge of the exposed area, and this was thought to be due to spreading from the margin of the shaded portion. More evidence on this point is needed. The conditions following rains seem to exert little influence on the spread of the angular leafspot of cotton.

Stewart and Leonard (1915, 1916) show conclusively that sucking insects are capable of inoculating young pear trees with *Bacillus amylovorus* when the bacteria are present upon the twigs at the immediate point of puncture or upon the mouth parts of the insects. Flies, etc., also have been determined to be a probable means of spread of the bacteria. To what extent these agents work together, in what other ways the bacteria may be spread from place to place to be inoculated by sucking insects, or to what extent inoculation may follow after insect punctures (since insect activity is lessened by rains, and the spread of the disease, according to these authors, is most noticeable after rainy periods) have not been determined. These authors state (1915, p. 121) that—

> On the other hand, it is to be noted that sucking bugs may be present in great numbers without the occurrence of much blight,

then describe two situations differing only in the amount of rainfall, and conclude that the resistant condition of the trees in the case where the drought prevailed lessened the blight as compared to a severe outbreak in the case of an abundant rainfall.

The work on Citrus canker is of particular interest and importance in this connection because the southeastern Citrus region is subjected to the tropical hurricanes, storms in which the wind blows highest and longest during rainfall and which above all others would serve for this purpose. Hasse determined the pathogenicity of her organism without wounds or injuries of the host plant, and Wolf concludes that rain and dew must serve as agents of local dissemination. If the action of a hurricane be combined with the motility of such a bacterium, in view of

the writer's results, this disease would undoubtedly be spread rapidly, as is noted by Stevens (1914).

The application of these conclusions to other diseases of such plants as walnut, cabbage, curcubits, oats, clover, sugar cane, and sweet corn, caused by motile bacteria which infect the aerial portions of the host, depends solely upon the prevalence of these meteorological conditions—that is, sufficient wind during rain to blow the water from plant to plant. It need not be from host to host, because other plants may serve as "stepping stones," as it were, provided the bacteria reached their host before the suspension became too dilute for infection. Their application is not at all limited to bacterial diseases, since there are many fungi, parasitic upon plants, having spores in sori, such as the Glomerellas and rusts, others having spores in pycnidia and in asci, where water is a necessary factor in their dissemination, and others having aerial spores, such as the Monilias and downy-mildews, where water is not necessary, yet in all probability would greatly augment infection by spreading the spores in the most favorable environment.

It is this factor which places all of these diseases in one theoretical class as regards control. Fungicides have not been generally accepted as a logical means of preventing bacterial diseases, though their efficiency should be the same as in the cases of potato-mildew, apple-scab, etc. A protective compound applied to the susceptible parts, active in the presence of water and of sufficient toxicity to kill the several bacteria quickly, would be expected to have the same result as in cases where sprays are recommended. In fact, Rolfs (1915, a) reports successful results with Bordeaux mixture in the prevention of this disease. Stewart (1913, a) mentions certain unpublished observations by Reddick in which a spray on the blossoms of pear lessened fireblight. Wolf and Massey (1914, p. 100), referring to Citrus canker, state:

> Very encouraging indications of successful control have been obtained by the use of each of these fungicides [Bordeaux mixture, ammoniacal copper carbonate, and soluble sulphur].

Sprays have been tried by some one or another in the case of almost every bacterial disease, and in the majority of instances negative results have been reported. This has developed the more or less current opinion that the theory of such methods is incorrect; whereas from these data one might conclude that the theory is correct, improper fungicides or ill-timed applications having been the cause of failures.

CONCLUSIONS

In conclusion and by way of summary the following points may be reiterated as of greatest importance:

(1) The methods of dissemination of many plant diseases, especially those caused by bacteria, are not satisfactorily described in the literature.

(2) In the case of the angular leafspot of cotton there is evidence of but little seed dissemination, though this is a probable factor in primary infection.

(3) Insects play a very unimportant part in the spread of this disease.

(4) Data have been obtained which point to the conclusion that wind-blown rain is an important factor.

(5) The records of the United States Weather Bureau show that such an agency is possible.

(6) Nothing in the literature precludes the assumption that such an agency may be effective in the dissemination of other similar diseases, while certain facts are on record which render it probable that this factor is of importance.

(7) The control measures used in the case of diseases disseminated by the wind which require water for infection may be expected to serve satisfactorily for this type of disease.

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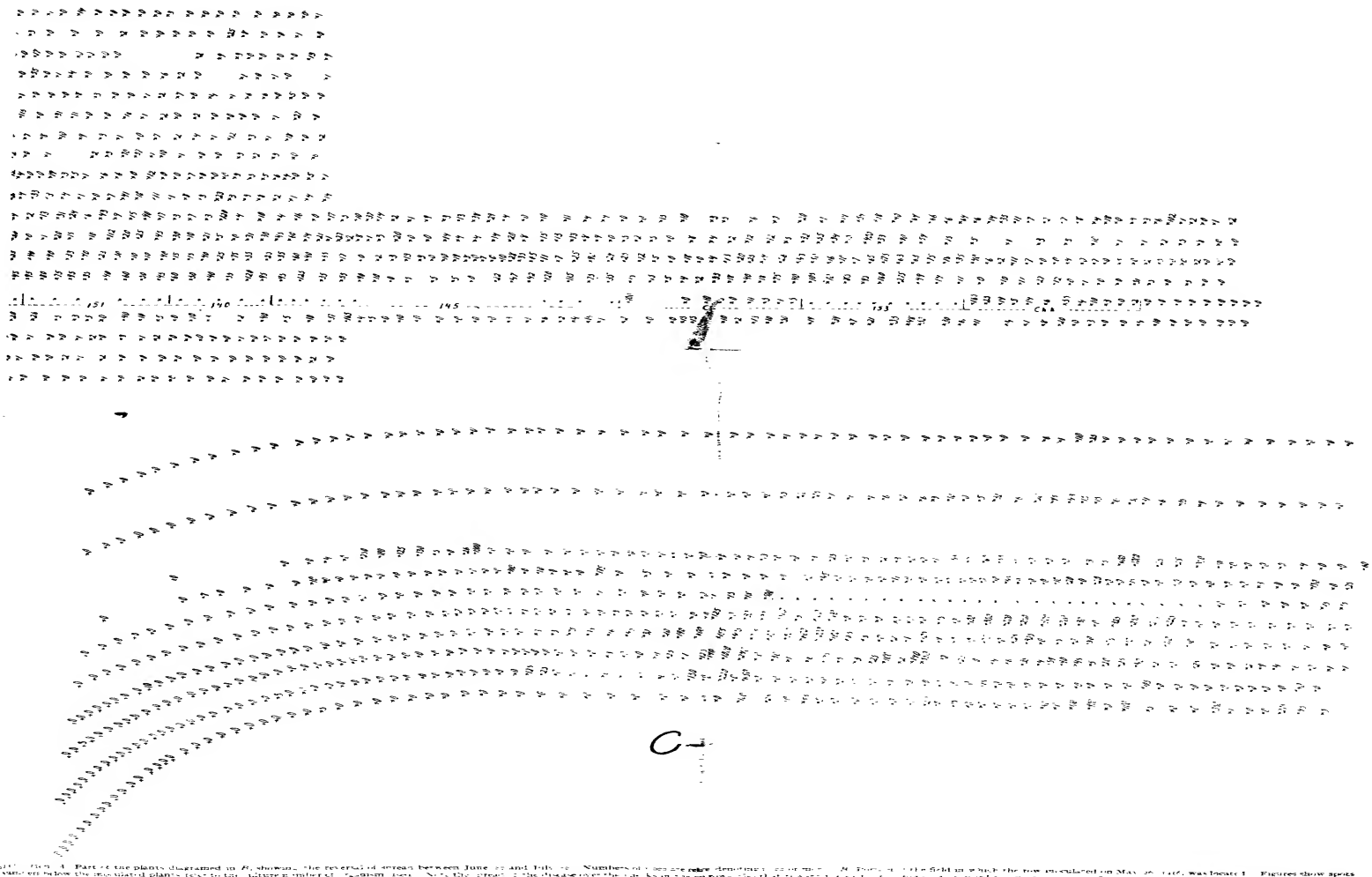
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